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Daniel Filipe Miranda Mendanha

UNDERSTANDING THE RELEVANCE OF ASTROCYTIC VESICULAR RELEASE IN GLIOBLASTOMA

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Doutor João Filipe Oliveira

e co-orientação do:

Doutor Bruno M. Costa

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DECLARAÇÃO

Nome: Daniel Filipe Miranda Mendanha

Endereço electrónico: mendanha@ecsaude.uminho.pt

Telefone: 913184484

Número do Cartão de Cidadão: 14398719

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Orientador:

Doutor João Filipe oliveira

Co-Orientador:

Doutor Bruno M.Costa

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Abstract

Glioblastomas (GBMs) are the most common and malignant type of glioma, the majority subtype of primary brain tumors. Despite the therapeutic advances over the past years, the median overall survival of patients is only 15 months after diagnosis. The high degree of heterogeneity between GBMs leads to an unpredictable clinical outcome. The variety of complex components in the tumor microenvironment has been associated with the aggressiveness of GBMs and with the inefficacy of treatments currently available. Astrocytes, the major glial cell type in the brain, are usually associated with the regulation of brain homeostasis, being involved in distinct regulatory processes, as angiogenesis or blood brain barrier (BBB) regulation. In GBM, astrocytes have been shown to secrete factors/proteins that may to regulate tumor growth, invasion, and progression. This secretion is performed, among others, by SNARE-dependent exocytosis, a mechanism impaired in astrocytes of the dnSNARE mouse model. By using this model, we explored the role of astrocytic exocytosis in the growth and invasion of GBM using *in vitro* and *in vivo* complementary approaches. We evaluated the effect of conditioned medium (CM), derived from wild – type (WT) and dnSNARE glial cultures, in the viability and migration of a glioma cell line. Moreover, using a syngeneic orthotopic glioma murine model (GL261), we evaluated the influence of astrocytic SNARE-dependent exocytosis in glioma growth *in vivo* and mice survival. The viability assays *in vitro* suggested a regulation of glioma cells by WT-derived CM, significantly increasing glioma cell viability, which was not significant when glioma cells were in contact with dnSNARE-derived CM. Concerning the *in vivo* results, our data suggest that the substances secreted by astrocytes appear to influence GBM behavior, leading to a decrease in mice survival. Interestingly, tumor size was similar in mice of both genotypes, suggesting other cancer hallmarks may be regulated in this GBM-astrocyte interaction. The results discussed in this thesis suggest that by releasing regulatory molecules, astrocytes might support GBM pathophysiology. The identification of the astrocyte-derived regulatory molecules may identify novel therapeutic targets for GBM treatment.

Resumo

Os glioblastomas (GBMs) são o tipo mais comum e maligno de gliomas, o maior sub-tipo de tumor primário no cérebro. Apesar dos avanços terapêuticos nos últimos anos, o tempo médio de sobrevivência dos pacientes depois de diagnosticados, é de apenas 15 meses. O elevado grau de heterogeneidade entre GBMs encontra-se relacionado com a imprevisibilidade no desenrolar desta doença. A variedade de células no microambiente tumoral tem sido associada com a agressividade apresentada por estes tumores e pela ineficácia dos tratamentos actualmente disponíveis. Os astrócitos, o principal tipo de célula da glia no cérebro, estão associados à regulação homeostática deste, estando envolvidos em processos como a angiogénese e a regulação da barreira hematoencefálica. Num contexto de GBM, os astrócitos secretam fatores/proteínas que podem regular o crescimento, invasão e progressão dos tumores. Esta secreção astrocítica, é mediada, entre outras, pelo complexo SNARE que regula a exocitose, e que se encontra inibido nos astrócitos do modelo de ratinho dnSNARE. Utilizando este modelo, foi explorado o papel da libertação vesicular astrocítica no crescimento e invasão dos glioblastomas utilizando abordagens complementares. O efeito do meio condicionado (MC), derivado de culturas de glia provenientes de animais controlo ou dnSNARE, foi testado na viabilidade e migração de uma linha celular de glioma. Complementarmente, usando um modelo ortotópico de glioblastoma (GL261), avaliamos a influência da exocitose mediada pelo complexo SNARE em astrócitos, no crescimento tumoral e na sobrevivência dos animais de ambos os genótipos. Os ensaios de viabilidade *in vitro* sugerem uma regulação das células de glioma pelo MC derivado de animais controlo, aumentando a viabilidade destas, o que não é significativo quando utilizado o MC derivado de culturas de glia dnSNARE. Relativamente aos resultados *in vivo*, estes sugerem que as substâncias secretadas pelos astrócitos podem influenciar o comportamento tumoral, levando a uma menor sobrevivência dos animais. Interessantemente, o volume tumoral foi similar entre genótipos, sugerindo que outras características tumorais podem ser mediadas por esta interação entre astrócitos e GBM. Os resultados discutidos durante esta tese sugerem que os astrócitos, libertando moléculas reguladoras, podem contribuir para as características do GBM. A identificação de moléculas derivadas de astrócitos podem apontar para novos alvos terapêuticos, no que diz respeito ao tratamento do GBM.

Table of Contents

Abbreviations List

ACM - Astrocyte Conditioned Medium

AD - Alzheimer´s Disease

ALS - Amyotrophic Lateral Sclerosis

Ang2 - Angiopoietin 2

ANOVA - Analysis of Variance

BBB - Blood Brain Barrier

BDNF - Brain-Derived Neurotrophic Factor

bFGF - basic Fibroblastic Growth Factor

BoNTs - Botulinum Neurotoxins

BrdU - 5-Bromo-2-DeoxyUridine

BSA - Bovine Serum Albumin

CM - Conditioned Medium

CNS - Central Nervous System

CSF-1 - Colony Stimulating Factor 1

Cx43 - Connexin43

DMEM - Dulbecco´s Modified Eagle Medium

DNA - Deoxyribonucleic Acid

DOX - Doxycycline

EC - Endothelial Cells

ECM - Extracellular Matrix

EGFP - Enhanced Green Fluorescent Protein

ET - Endothelin

FBS - Fetal Bovine Serum

GBM - Glioblastoma

GDF-15 - Growth Differentiation Factor 15

GDNF - Glial Derived Neurotrophic Factor

GFAP - Glial Fibrillary Acidic Protein

GFP – Green Fluorescence Protein

GSC- Glioma Stem Cells

H&E - Hematoxylin and Eosin

HBSS - Hank´s Balanced Salt Solution

HIF - Hypoxia Inducible Factor

IGF-1 - Insulin-like Growth Factor 1

IL-10 - Interleukin 10

IL-6 - Interleukin 6

KPS - Karnofsky Performance Status

M1 - Macrophages Type 1

M2 - Macrophages type 2

MCP-1 - Monocyte Chemoattractant Protein-1

M-CSF - Macrophage Colony-Stimulating Factor

MMP - Matrix Metalloprotease

MT1-MMP - Membrane type 1 metalloprotease

OS - Overall Survival

PCR – Polymerase Chain Reaction

PD - Parkinson´s Disease

PFA - Paraformaldehyde

PVN - Perivascular Niche

ROS - Reactive Oxygen Species

RPM - Rotations per Minute

RT - Room Temperature

SDS - Sodium Dodecyl Sulfate

SNAP - Synaptossomal Associated Protein

sRNAs - small Non Coding RNAs

STI1 - Stress-Inducible Protein 1

STX1A – Syntaxin Protein 1

TAM - Tumor Associated Macrophage

TEM - Tie-2 Expressing Monocyte

TGF-β - Transforming Growth Factor Beta

TLR2 - Toll Like Receptor 2

TMZ - Temozolomide

TNC - Tenascin-C

TNF-α - Tumor Necrosis Factor Alpha

tTA - Tetracycline Transactivator

VAMP - Vesicular Associated Membrane Protein

VAMP 2 - Synaptobrevin 2

VEGF - Vascular Endothelial Growth Factor

WHO - World Health Organization

WT - Wild-type

Figures List

Table List

Chapter 1 – Introduction

1 Introduction

In the current century, cancer remains one of the major health problems, affecting people of every socioeconomic status around the world. According to World Health Organization (WHO), 14.1 million of new cancer cases were diagnosed in 2012. Despite the advances in cancer therapies, as chemotherapy and radiotherapy, 8.2 million cancer deaths were accounted in 2012 (Ferlay et al., 2015).

Cancer can be defined as uncontrolled cell growth with invasive/metastatic potential resultant from the accumulation of molecular alterations, such as deoxyribonucleic acid (DNA) mutations, copy number aberrations, chromosomal rearrangements and epigenetic modifications (responsible for gene expression regulation) (McLendon et al., 2008). These events are usually associated with the activation of oncogenes and deactivation of tumor suppressor genes. These genes are responsible for the regulation of cell proliferation, survival and differentiation, being necessary an alteration in both gene types for a neoplastic transformation. The deregulation of key signaling pathways is associated with genetic alterations neoplastic cells, that result in specific cancer features (*e.g.* proliferation, invasion, angiogenesis, *etc.*). In addition to the genetic alterations, the cellular microenvironment is also crucial for a neoplastic profile, where, for instance, increased secretion of growth factors can constitutively activate key pathways for cell proliferation (Hanahan and Weinberg, 2011).

With the continuous growth of knowledge about cancer dynamics, it is well understood that tumors are not homogeneous masses of proliferating cancer cells. In fact, they are complex tissues composed by a large variety of cells, from parenchyma to immune cells, that together with cancer cells establish a large range of interactions. Thus, the advances in cancer therapeutics cannot only pass through the target of neoplastic cells, being necessary to understand and stop the contribution of the tumor microenvironment to tumorigenesis. In this scope, Hanahan and Weinberg proposed the ten cancer hallmarks (Figure 1.1), that include: i) evading growth suppressors; ii) avoiding immune destruction; iii) enabling replicative immortality; iv) tumor promoting inflammation; v) activating invasion & metastasis; vi) inducing angiogenesis; vii) genome instability & mutation; viii) resistance to cell death; ix) deregulating cellular energetics and x) sustaining proliferative signaling (Hanahan and Weinberg, 2011).

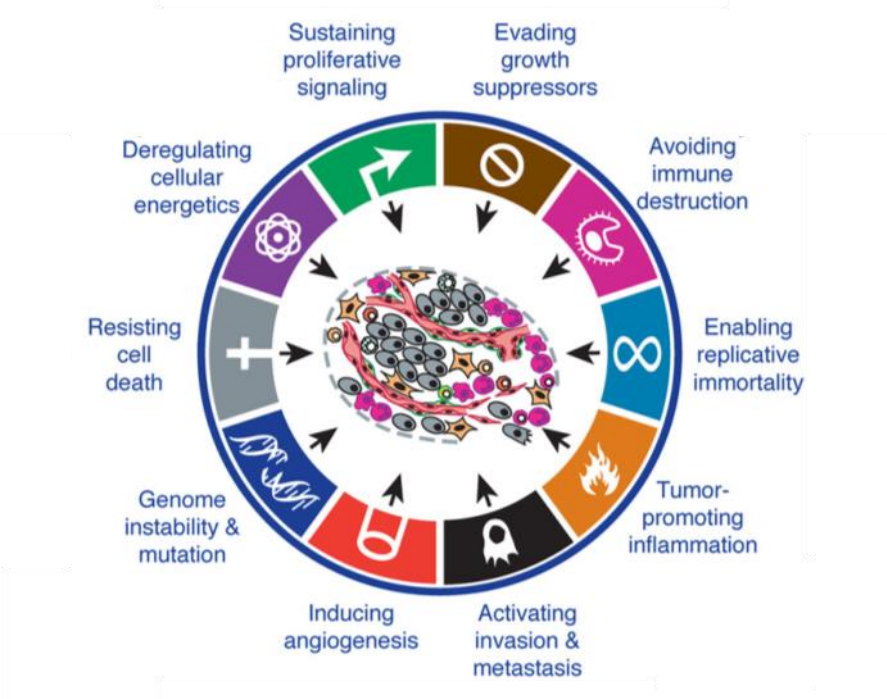

Figure 1.1 – The hallmarks of cancer – Cancer cells present specific capabilities that enhance tumor growth and metastatic dissemination (Adapted from (Hanahan and Weinberg, 2011)).

1.1 Primary brain tumors

The central nervous system (CNS) can present several tumor subtypes, which can be grossly separated in benign and malignant entities (Ostgathe et al., 2010). Tumors that have their origin in brain cells are classified as primary brain tumors, in contrast to cancer cells from a different origin that spread into the brain, commonly designated as brain metastases. Primary brain tumors present a low incidence between the primary tumors, only 2 %, nonetheless presented a high mortality rate (Buckner et al., 2007, Louis et al., 2007).

In 2007, the WHO published its 4th edition updating the classification of CNS tumors. According to this manuscript, more than 100 different types were already described, regarding their origin, localization and histopathological features. By measuring these distinct characteristics WHO created a classification that grades CNS tumors in a malignant scale, being gliomas the most common form of brain tumor in CNS (Louis et al., 2007, Ostrom et al., 2014). An update version of CNS tumors classification was published this year, where for the first time, tumors were classified not only by their histological features but also by the distinct molecular components of tumors, resulting in the classification of new entities (Louis et al., 2016).

1.1.1 Glioma

The origin of gliomas is still unclear, being crucial to understand how a series of molecular alterations that began in a couple of cells can result in a devastating disease. Nowadays, two major hypothesis emerge regarding glioma origin. The first one postulates that the accumulation of mutations and alterations in differentiated glial cells, as astrocytes or oligodendrocytes, leads to a dedifferentiation of those cells, acquiring a rapid proliferative and neoplastic profile. The second hypothesis, proposes that carcinogenic cells have their origin in progenitor undifferentiated cells, present in specific niches of the brain, that undergo molecular alterations resulting in neoplastic transformation (known as glioma initiating cells) (reviewed in Gonçalves et al., 2013). In fact, supporting this hypothesis are the glioma stem cells (GSC) that can be found in tumors, presenting a self-renewal capacity and high replicative potential, and which are able to promote the development of gliomas (Nguyen et al., 2012, Sampetrean and Saya, 2013). For the majority of gliomas, no underlying carcinogenic have been identified, being the exposure to high-dose of ionizing radiation the only well-established environmental risk factor established. Although some epidemiological studies in glioma have been published, the data regarding other environmental risk factors are still inconclusive (Bondy et al., 2008, Ohgaki, 2009).

Gliomas represent approximately 80 % of all malignant brain tumors (Ostrom et al., 2014). With several specific pathological and immunohistochemical characteristics, gliomas are usually classified considering the type of glial characteristics they present (Figure 1.2). Therefore, it is usual to separate gliomas in: astrocytomas (similarities with astrocytes); oligodendrogliomas (similarities with oligodendrocytes); oligoastrocytomas (hold mixed characteristics from astrocytes and oligodendrocytes) and ependymomas (present similar features to ependymal cells) (Louis et al., 2007). Considering all the resemblances between these cells, it is usually accepted that glioma subtypes have their origin in the specific glial cell subtypes or their precursors. Being one subtype of primary brain tumors, gliomas are also classified according to their malignancy in four distinct grades (I-IV) by the WHO. Grade I gliomas are designated as benign tumors with a low proliferative potential, having the possibility of cure by surgical resection. Tumors designated as grade II present an infiltrative nature and are already classified as malignant tumors although, together with grade I tumors, they are considered low grade gliomas. Usually, the grade II tumors progress to high grade tumors, as for example, the low-grade diffuse astrocytoma (II) that is able to progress to glioblastoma (IV). Grade III gliomas present histological evidences of malignancy, as is the case of nuclear atypia and mitotic activity. Finally, the most malignant gliomas are classified with grade IV and are usually associated with a rapid pre and post-operative disease evolution

and a fatal outcome for the patient, being designated glioblastomas (Louis et al., 2007, Ostrom et al., 2014).

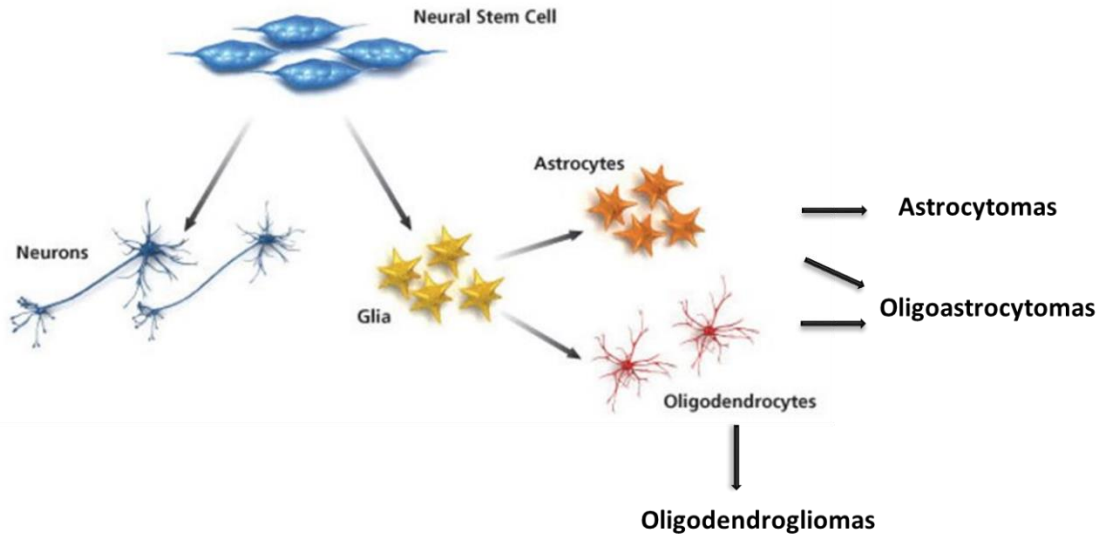

Figure 1.2 - Classification of gliomas based in their origin and molecular characteristics – Gliomas can be classified considering the histological similarity to the major type of glial cells. Astrocytomas have similar characteristics to astrocytes and oligodendrogliomas present similar characteristics to oligodendrocytes. Oligoastrocytomas, have features that are present in both glial cell types.

1.1.2 Glioblastoma (GBM)

Glioblastoma (GBM) is the most common and malignant primary brain tumor, being the most common subtype of glioma (54.7% of all gliomas) (Ostrom et al., 2014). According to the WHO, GBM presents a grade IV classification, being represented by nuclear atypia, increased mitotic activity, microvascular proliferation and tissue necrosis. The tumor mass of GBM is characterized by its poor delineation and for having a high degree of regional heterogeneity. In the peripheral zones of the tumor it is common to find highly proliferating cancer cells, where the center of the tumor is mainly constituted by necrotic tissue (Inda et al., 2014, Hambardzumyan and Bergers, 2015). Despite being a cancer with low metastatic capacity, GBM is a highly invasive tumor especially along myelinated brain structures. The invasiveness of cells is the main reason for the poor efficacy of therapies, due to their ability to escape from surgical resection and radiotherapy (that target the main tumor mass) originating local recurrences (Louis et al., 2007).

In 1940, Hans-Joachim Scherer, a pioneer in glioma research, established for the first time a difference between the primary and secondary GBM (Scherer, 1940). Nowadays, we known that approximately 90 % of GBM cases are primary, meaning that they arise *de novo*, usually in elderly patients without a clinical or histological evidence of a lower grade glioma. Patients with GBM are usually asymptomatic until a late course of the disease, when the first clinical signs appear (*e.g.* headache and nausea) derived from the intracranial pressure rise caused by the tumor mass size, complicating the early detection and treatment (Wen and Kesari, 2008). Secondary GBM represent the remaining 10 % of the cases, progress from lower grade astrocytomas, and manifest in younger patients presenting a better prognosis. Histologically, primary and secondary GBM, are virtually indistinguishable, but they present several differences regarding the genetic and epigenetic profile (Ohgaki and Kleihues, 2007, 2013).

Despite the therapeutic advances over the past 20 years, the median overall survival of patients is only 15 months after diagnosis and treatment. In fact, all the treatments available are mostly palliative (Stupp et al., 2005, Wen and Kesari, 2008). The surgical resection is in the first line of treatments against GBM, corresponding to the resection of the maximal volume of tumor mass without putting in risk the CNS system activity of the patient. Usually, the surgical resection is followed by a radiotherapy treatment on a dose schedule of 60 Gy administered in 2.0 Gy per fraction (Malmstrom et al., 2012, Lacroix and Toms, 2014). Several advances in chemotherapy have also emerged in the last decade, being a standard treatment the use of the alkylating agent temozolomide (TMZ) in conjugation with radiotherapy (Stupp et al., 2005). Other therapeutics have also been used in the treatment of this disease, including bevacizumab, a new anti-angiogenic drug, that targets the high vascularization of these tumors by bindings to the vascular endothelial growth factor (VEGF), neutralizing its biological activity and leading to a deficient angiogenesis that slows GBM progression (Friedman et al., 2009).

1.1.3 Glioma microenvironment

In the recent years, the interest in glioma microenvironment has arose, and several studies showed the importance of parenchyma cells in the course of the pathology. There is a current recognition that gliomas are complex tumors composed of neoplastic and non-neoplastic cells, being virtually each type individually able to contribute for cancer formation, progression and or response to treatment. The microenvironment of glioma is usually composed by different non-neoplastic cell types including fibroblasts, endothelial cells and immune system cells (Hambardzumyan et al., 2015). However, the interaction between brain-resident and infiltrating cells in the pathology of primary and metastatic brain

tumors is still poorly understood (Lorger, 2012). To help understanding the complexity of glioma microenvironment, Hambardzumyan and Bergers proposed a compartmentalization of the tumor microenvironment into three anatomically distinct regions, designated tumor niches (perivascular niche (PVN), hypoxic niche, invasive niche; Figure 1.3). In the tumor microenvironmental niches, tumor and stroma cells interact via direct cell contact or paracrine signaling to ensure maintenance, growth and protection of tumor and cancer stem cells (Hambardzumyan and Bergers, 2015).

The PVN presents a multicellular structure composed by several non-neoplastic cells already present in the brain or recruited from the periphery. The main function of this niche is to provide a supportive environment for cancer cells. The second GBM niche is the hypoxic niche, created by the abnormal vascular function in GBM, which leads to a deficient oxygen deliver within the tumor. Usually present in the center of the tumor mass, the niche is characterized by several necrotic areas responsible for the release of hypoxia inducible factors (HIF-1α and HIF-2α), leading to the expansion of GSC and the recruitment of innate immune cells. The last niche is usually found in more peripheral tumor zones, and it is designated as invasive niche. This niche is characterized by a large population of non-neoplastic cells that contribute to the invasiveness of glioma cells. Although astrocytes and pericytes are partially detached in these particular areas, it is possible to see an increased functional vasculature, that can be used to invade different brain areas. Moreover, it is important to understand that niches are non-static and develop several alterations with the course of the disease (Hambardzumyan and Bergers, 2015). Over the last years, studies targeting the microenvironment emerged, mostly due to the failure of treatments targeting the neoplastic cells. However, it is still necessary to understand how each of the different cell types present in the tumor can influence the disease progression.

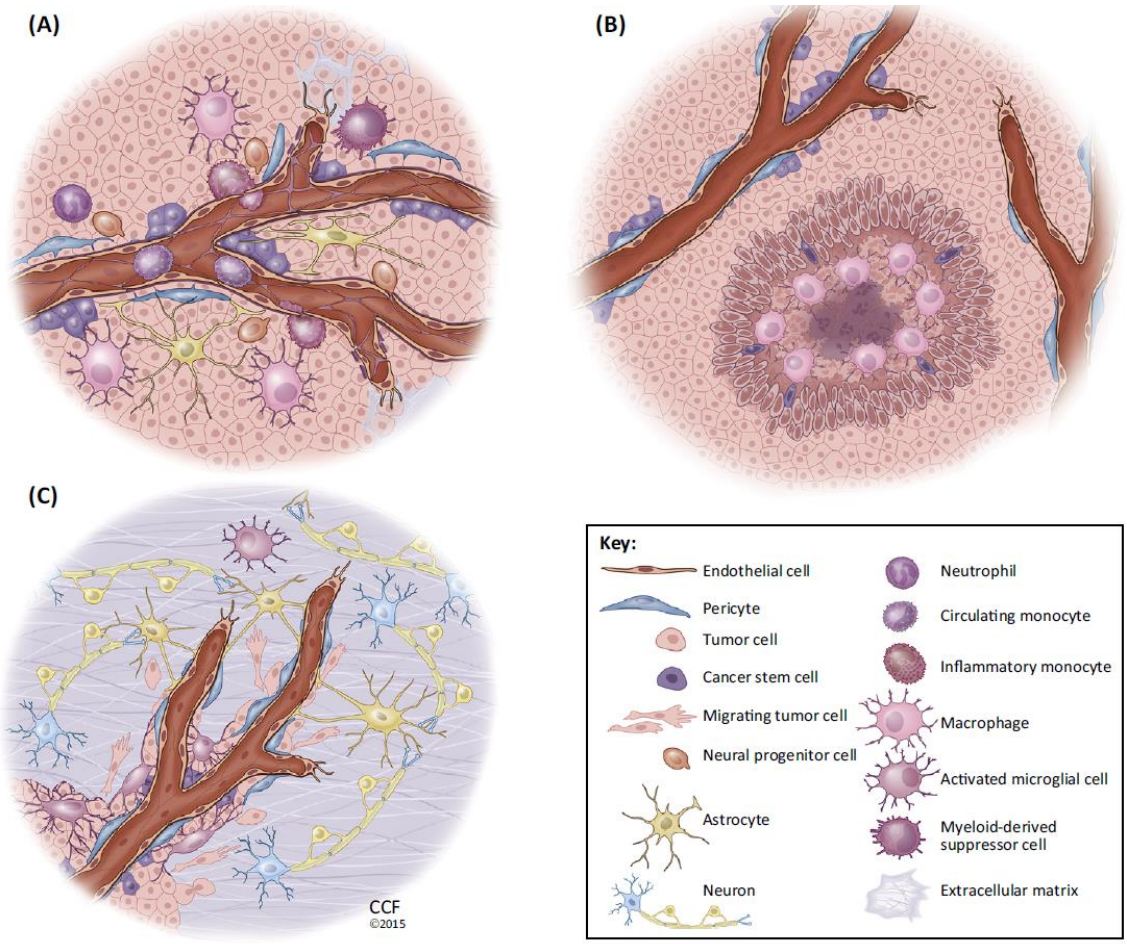

Figure 1.3. - Tumor niches of Glioblastoma - Glioblastomas can present different niches, that present specific features and different intra-cellular composition. **(A)** Perivascular GBM niche – present a multicellular structure composed for several non-neoplastic cells (*e.g.* pericytes, microglia, astrocytes), providing a supportive environment for neoplastic cells. **(B)** Hypoxic GBM niche – present in the center of a tumor mass with a considerable volume, do not present a larger cellular diversity due to the lack of oxygen. Is usually associated for the release of factors that attract cells of the immune system. **(C)** Invasive GBM niche – similar to niche (A), also present a rich diversity of non-neoplastic cells, that release enhancer invasion factors. Present a rich and functional vascularization that can be used by glioblastoma cells to invade (Hambardzumyan and Bergers, 2015).

The complex role of the tumor microenvironment in the progression of brain tumors is still poorly understood, and only recently the scientific field has started to decode the interaction between stromal and cancer cells. Microglia and macrophages have been the primary targets of new studies for their important immunological role and, in fact, they present a significant role in the disease progression. However, it is important to understand how the different components of the tumor microenvironment individually and collectively contribute to the tumor. In the case of primary brain tumors, astrocytes, the

main glial cell in the brain, can play a crucial role in the disease development. Since this project focus on the astrocytic modulation of GBM, the next sub-chapters will summarize the functions that have already been attributed to non-neoplastic cells present in the brain tumor microenvironment, and a new sub-chapter will be dedicated to astrocytes and their relations to GBM.

Microglia and tumor associated macrophages (TAMs)

Microglia are a type of mononuclear cells that are distributed throughout the brain and act as immune effector cells of the CNS, being considered the resident macrophages of CNS (Hambardzumyan et al., 2015). In neuropathological conditions, as GBM, the blood brain barrier (BBB) is disrupted, resulting in an infiltration of monocytes from the periphery (Hambardzumyan et al., 2015). After entering the CNS, monocytes are able to differentiate into tumor-associated macrophages (TAMs), which present a high level of similarity with resident microglia. In fact, TAMs and microglia cells together, constitute approximately 30 to 40 % of the cells in GBM (Charles et al., 2011, Hambardzumyan and Bergers, 2015).

Microglia and TAMs react to cancer cells, being these cell types already found accumulated around a single metastatic cancer cell, acquiring an amoeboid morphology. The migration of microglia and TAMs into glioma cells results from the release of chemo attractive factors by glioma cells, such as: monocyte chemoattractant protein-1 (MCP-1); glial-derived neurotrophic factor (GDNF) and colony stimulating factor 1 (CSF-1). Glioma cells can also release factors that induce a shift in microglia and macrophages phenotype, as is the case of macrophage colony-stimulating factor (M-CSF) (Pyonteck et al., 2013). Macrophages can be simplistically separated based in two distinct profiles: type 1 macrophages (M1), that present a pro-inflammatory profile and are usually associated with the release of anti-tumorigenic substances; and type 2 macrophages (M2), that have an anti-inflammatory profile, presenting pro-tumorigenic characteristics. TAMs are commonly associated with a M2 profile, although presenting some differences. The regulation and manipulation of this profile switch has been proposed as a possible therapeutic target for gliomas (reviewed in Hambardzumyan et al., 2015).

The accumulation of microglia and TAMs in and around glioma cells results in a direct interaction between them, resulting in the promotion of glioma growth and invasion. In 2009, Markovic and co-workers, established that a reduction in microglia number in a transgenic mice model, was sufficient to attenuate glioma growth (Markovic et al., 2009). Microglia and TAMs around the tumor present a high expression of stress-inducible protein 1 (STI1), which upon secretion promotes glioma growth (da Fonseca et al., 2014).

High grade gliomas are severely invasive and a large number of studies already attributed some of this invasive capacity to the role of microglia and macrophages. The release of factors by glioma cells, regulating the pro-tumorigenic profile of microglia, leads to an increase in interleukyn-6 (IL-6) secretion by microglia, promoting the invasive capacity of glioma cells (Saederup et al., 2010). Transforming growth factor-β (TGF-β) released by microglia can also increase glioma migration through a process involving increased integrin function and expression (Wick et al., 2001). Besides that, TGF-β induces the expression of matrix metalloprotease 2 (MMP2) that acts in the extracellular matrix (ECM) facilitating the glioma invasion (Markovic et al., 2005). In fact, a recent study showed that glioma cells release versican, an endogenous ligand that triggers the toll like receptor 2 (TLR2) signaling. An increased TLR2 expression is able to switch the microglia to a pro-tumorigenic phenotype resulting in an upregulation of matrix metalloprotease 9 (MMP-9) and membrane type – 1 matrix metalloproteinase (MT1-MMP), which in turn can lead to changes in ECM and ultimately to an increase in glioma growth and invasion (Vinnakota et al., 2013, Hu et al., 2014, Hambardzumyan and Bergers, 2015).

A different population of myeloid cells that derives from circulatory monocytes, are the Tie-2 expressing monocytes (TEMs). This subpopulation, which only accounts for approximately 7 % of blood mononuclear cells, has been associated with a high impact in tumor angiogenesis (De Palma et al., 2005, Venneri et al., 2007). Similarly to TAMs, when they reach the tumor, after crossing the BBB, they strongly polarize to a M2 activation state, secreting basic fibroblastic growth factor (bFGF) that induces a pro-angiogenic activity (Lorger, 2012). More recently, it was suggested that TEMs play an important role in glioma anti-vascular therapies, by promoting the invasion of cancer cells after the treatment. The use of Bevacizumab, since 2009, an anti-VEGF agent, did not reach the level of efficacy expected. In 2016, Cortes-Santiago and co-workers, claimed for the first time, that TEMs can be responsible for this inefficacy. They suggested that using an agent against angiopoietin 2 (Ang2) combined with anti-VEGF therapy, may result in the improvement of the treatment and in less glioma recurrences (Cortes-Santiago et al., 2016).

Endothelial cells

The high vascularization characteristic of gliomas, has in its base the endothelial cells (EC) that constitute the blood vessels. These cells have emerged as critical participants in the progression of brain tumors, not only by allowing a constant flux of oxygen and nutrients to tumor cells, but also by the direct communication with glioblastoma stem cells (GSC). The secreted factors by ECs contribute to the GSC maintenance of their stem cell-like characteristics (Charles et al., 2011). In 2010, Charles and his co-

workers suggested that nitric oxide released by ECs activates the Notch signaling in cancer cells, contributing to the maintenance of the stem cell-like state (Charles et al., 2010). Moreover, the increase of blood vessels or endothelial cells in orthotropic brain tumor was associated with the increase of the self-renewing population, resulting in a faster tumor growth (Bonavia et al., 2011).

Endothelial cells ,together with pericytes and astrocytes, are the main cell components of BBB, a neurovascular unit responsible for the regulation of dynamic exchanges between bloodstream and the brain, giving the brain an immune-privileged position (Abbott, 2013). In primary brain tumors BBB is disrupted mainly by loss of properties by ECs and is responsible for the income of innate immune system cells in the brain that, as previously mentioned, contribute to the progression of brain cancer (Hambardzumyan and Bergers, 2015).

Pericytes

The strong and abnormal angiogenesis is one of the main GBM features, where is common to find disorganized and leaky blood vessels (Hambardzumyan and Bergers, 2015). This feature is usually attributed to the detachment of pericytes from the vessels, caused by the increased concentration of VEGF in the tumor microenvironment. Pericytes are perivascular cells with contractible capacity that support blood vessels and promote vascular maturation, being usually known for the control of dynamic processes in vasculature (Cleaver and Melton, 2003, Bergers and Song, 2005). In the context of tumor development, recruitment of pericytes to tumor core is crucial for structural stability and survival of endothelial cells. Nowadays, it is believed that glioma cells release HIF-1α, a chemoattractant for pericytes progenitor cells, which results in the promotion of angiogenesis and glioma neovascularization, a feature associated to malignant gliomas (Chekenya et al., 2002, De Palma et al., 2005).

1.2 Astrocytes

More than 150 years passed since Rudolf Virchow introduced for the first time the concept of neuroglia, which he designated as the brain connective tissue, assuming years later that the tissue “also contains a certain number of cellular elements” (Virchow, 1856). The second part of 19th century was rich in advances in cellular histology, with different glial cells being described. In 1893, Michael von Lenhossek proposed for the first time the term “astrocyte”, further spliced in fibrous and proplasmatic regarding their localization, white and grey matter, respectively (Lenhossék, 1893). Our knowledge on the

properties and diversity of neuroglial cells have dramatically increased. Even though, many years already passed and the whole range of actions in the CNS and their role on brain diseases is still far away from totally understood (Kettenmann and Verkhratsky, 2008).

Defining an astrocyte is not a simple task but it has been accepted that astrocytes are heterogeneous cells with a star-shaped morphology, with extending numerous processes that surround neighbor neurons and blood vessels. The most defining characteristic of astrocytes is the expression of glial fibrils, known as intermediate filaments (Wang and Bordey, 2008). Perhaps, because of the lack of ability to form action potentials, astrocytes were for many years saw as the "*ugly duck*" by the scientific community. However, with brain research development and with several emerging functions being associated to these cells nowadays, thinking of astrocytes merely as neuron supporting cells is immensely reductive.

In 1909, less than 20 years after astrocytes been described, Held and his team proves the ability of astrocytes to secret molecules that is known to be crucial for brain homeostasis (Held, 1909). Astrocytes have been already associated to several brain functions, being the synthesis of proteins and adhesion molecules that compose the ECM one of them. However, these cells are also able to synthetize and secrete proteolytic enzymes, as MMPs that play a vital role in ECM degradation and remodeling (Muir et al., 2002). Their role in angiogenesis and BBB induction and maintenance are other physiological aspects attributed to astrocytes action. A better understanding of angiogenesis and of the dynamics between astrocytes and endothelial cells (to regulate BBB stability and permeability) is critical for understanding the process of tumorigenesis and neurogenesis. In fact, astrocytes act as a part of a neuroglial secretory network, that can be defined as *gliocrine* system of the CNS (Vardjan and Zorec, 2015). With a wide range of substances, some of the astroglia-derived secretory substances are: neurotransmitters and their precursors; hormones and peptides; eicosanoids, scavenger reactive oxygen species (ROS); growth factors; "*plastic*" factors and pathological molecules, as inflammatory factors (Verkhratsky et al., 2016). The release of all these substances can occur by distinct pathways, including diffusion through plasmalemmal pore/channels; extrusion through plasmalemmal transporters and also by vesicle-based exocytosis (Figure 1.4). Regarding the release by exocytosis, different vesicles have already been identified including: small vesicles, dense core vesicles, lysosomes, exosomes and ectosomes. The vesicles released by astrocytes can have different fusion events with the plasmatic membrane and, more importantly, different contents. The evolutionary conserved family of SNARE proteins are the foundation of this secretory mechanism. The family can be divided in two categories, R-SNAREs and Q-SNAREs, being associated with the vesicular membrane or with plasma membrane

proteins, respectively (Jahn and Scheller, 2006). In vesicular release events, there is an increase in intracellular calcium (Ca^{2+}) levels, leading to the association of vesicle proteins to membrane proteins (syntaxins), and to the formation of a ternary SNARE complex. The proteins of SNARE complex, namely synaptobrevin (VAMP), syntaxin (STX) and synaptossomal associated protein (SNAP), create a 4 α-helical bundle (SNAREpin), that allows the fusion of vesicular and plasma membranes (Sutton et al., 1998, Hamilton and Attwell, 2010, Verkhratsky et al., 2016). The SNARE complex, is not exclusive of astrocytes, and other brain cells like neurons also use the complex to release substances. Interestingly, Ulloa and his co-workers in 2015, inhibiting the SNARE protein STX1 in glioblastoma cells, showed a significant decrease of proliferation and invasion. This work supported the importance of autocrine signaling for glioblastoma cells, but also that the autocrine signaling can be targeted using the complex SNARE (Ulloa et al., 2015).

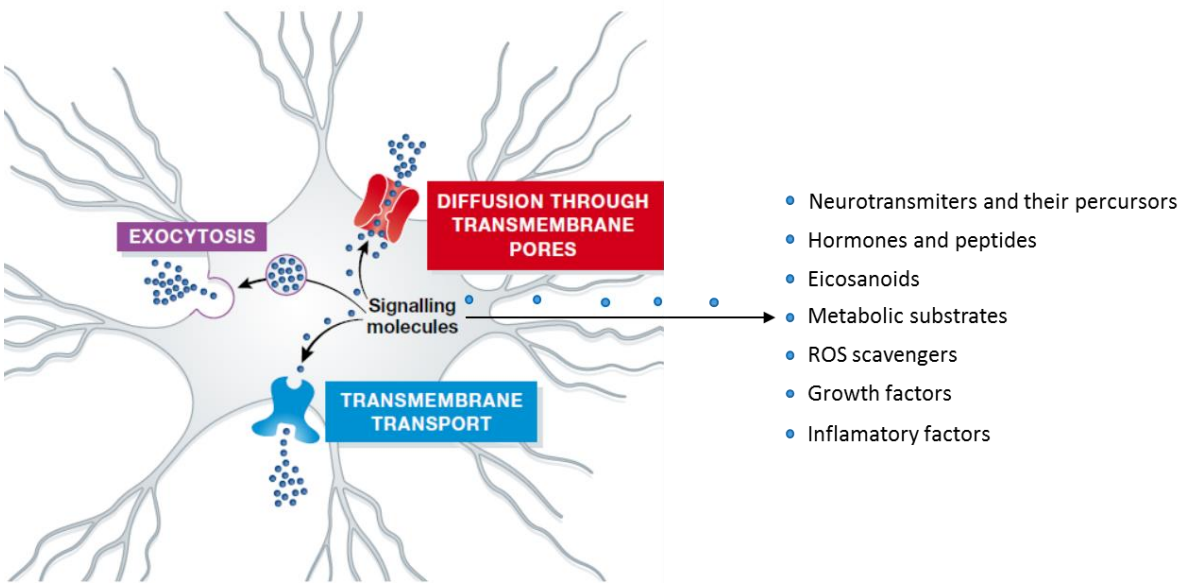

Figure 1.4 - Secretory pathways and secreted substances by astrocytes - Astrocytes present three main secretory pathways responsible for the release of several substances crucial for brain homeostasis. Between the secreted molecules is usually to found hormones and peptides, metabolic substrates, growth factors and inflammatory factors (Adapted from (Verkhratsky et al., 2016)).

Astrocytes present a significant importance in the response to brain injuries, usually characterized by bleeding and an intense local inflammation. Astrocytes closely positioned to injury, respond to it becoming hypertrophic and forming a scar in conjugation with other brain cells (*e.g.* fibroblasts), that isolate the damaged tissue from the remaining healthy brain tissue (Cregg et al., 2014). Astrocytes near

the injury site not only upregulate their expression of glial fibrillary acidic protein (GFAP), but also several proteins and transporters, by a process known as astrogliosis (Vijayan et al., 1990, Filous and Silver, 2016). Astrocytes are involved in several neuropathologies playing an important role in the development and progression of the disease. Alzheimer´s disease (AD) is a progressive neurodegenerative disease, characterized by the presence of amyloid beta plaques and neurofibrillary tau tangles. The main component of beta plaques, β-amyloid, is a neurotoxic agent that promotes the response of astrocytes and microglia, leading to the release of inflammation promoting mediators, potentially neurotoxic. In case of injury, the release of these substances is beneficial for brain damage repair, but in a case of a chronic disorder a constant state of activation by astrocytes and microglia leads to a chronic inflammation that contributes to secondary nerve damages. It is currently described that astrocytes activated by β-amyloid release pro-inflammatory cytokines and ROS, contribute to neuronal damage, a hallmark of AD. (Markiewicz and Lukomska, 2006, Filous and Silver, 2016). Interestingly, gap junctions between astrocytes are altered in AD, namely by the observable increase in the gap junctional protein connexin 43 (Cx43)(Nagy et al., 1996). Moreover, gap junction proteins expression has been associated with an increased release of glutamate and ATP, resulting in a glutamatergic cytotoxicity, that leads to neuronal damages (Nakase and Naus, 2004).

Astrocytes are also involved in other progressive neurodegenerative disorders, such as amyotrophic lateral sclerosis (ALS), characterized by the death of motor neurons in cerebral cortex (Vargas and Johnson, 2010). In ALS, astrocytes have an upregulated expression of the inflammatory cytokine transforming growth factor-β1 (TGF-β1), which stops microglia and T cells production of insulin-like growth factor 1 (IGF-1), resulting in a loss of inflammatory-mediated neuroprotection and, consequently, in a faster progression of the disease (Endo et al., 2015). Additionally, Parkinson´s disease (PD), characterized by the loss of dopaminergic neurons in the substance nigra and the presence of Lewy bodies (aggregates of α-synuclein), astrocytes also have an important role on the disease progression. The endocytosis of α-synuclein by astrocytes results in the upregulation of pro-inflammatory cytokines and chemokines, resulting in a constant inflammatory state prejudicial for neurons (Lee et al., 2010, Filous and Silver, 2016). Furthermore, astrocytes are also involved in epilepsy, where the proliferation of reactive astrocytes is a common feature in temporal lobe epilepsy. An astrocytic dysfunction in epilepsy, with alterations in channel expression and dysfunctional gap junctions, leads to an increase in the number of seizures and to glutamate cytotoxicity (Filous and Silver, 2016).

The astrocytic deregulation is the basis of several brain disorders, usually contributing to a constant state of inflammation. Although essential for brain homeostasis, the secretory capacity of astrocytes is

usually altered in neurological disorders, leading to an excessive or deficient release of substances that disrupt the brain homeostasis. The unbalanced release of chemokines and cytokines, and also the disruption of gap junctions between astrocytes, have been already associated to distinct disorders (O'Brien et al., 2014). New therapeutic approaches targeting dysfunctional astrocytes and reducing its inflammatory state, can emerge in the next years. In fact, it is essential to understand not only how dysfunctional astrocytes contribute to several brain diseases, but also how are astrocytic mechanisms relevant for disease progression. In GBM, astrocytes are associated as tumor supportive cells but the underlying mechanisms of this support are still poorly understood. In the next chapter, a literature background will be presented about the interactions between astrocytes and tumor cells.

1.3 Role of astrocytes in gliomas and brain metastases

Since several brain cell types have an impact on the development of diseases, it is difficult to disclose the role of astrocytes, the major glial cell in the brain, in the disease. Despite the emerging studies establishing a connection between these glial cells and glioma cells, the consequences of these interactions remain unclear, especially *in vivo*. In cases of brain pathology or injury, BBB damage and cancer, astrocytes undergo several morphological changes being this process usually called reactive astrogliosis (Wilhelmsson et al., 2006, O'Brien et al., 2014). In this state, astrocytes present an upregulation of GFAP and vimentin, as well as several growth factors, inflammatory cytokines and ECM proteins. In sporadic lesion cases, the physiological alterations followed by alterations in astrocytic secretion can present benefits for the recovery of the brain damage (Sofroniew, 2009). However, in cancer, the astrocytes surrounding the cancer cells start to secrete several proteins that can help the growth and spread of the cancer. In fact, astrocytes and some of their secreted proteins, have already been proposed to enhance cancer progression (O'Brien et al., 2013). Astrocyte-derived signaling was reported to modulate important aspects of brain cancer progression, such as cell proliferation and tumor invasiveness, which are detailed below.

Astrocytes were reported in several studies to promote proliferation of cancer cells. *In vitro* studies showed that the presence of astrocytes, astrocyte-conditioned media (ACM) or specific growth factors secreted by reactive astrocytes, triggers an increase in cell proliferation in different brain metastasis (Placone et al., 2016). Diverse molecules secreted by reactive astrocytes, as IL-6, IGF-1 or TGF-β, have been independently associated to a proliferation increase in different glioma cell lines (Li et al., 2010, Roth et al., 2010). In studies from Li and co-workers, IL-6 was responsible for an increase of 25% of cell

proliferation in a human glioma cell line U87MG (Li et al., 2010). In a different study, growth differentiation factor 15 (GDF-15), a factor upregulated in reactive astrocytes, was also able to increase cell proliferation (Roth et al., 2010). Besides the effects observed using glioma cell lines, astrocytes were also related with the proliferation of metastatic, breast and lung cancer cells. In fact, studies using these cancer cell lines co-cultured either with astrocytes, or exposed to ACM, present increased proliferative rates (Sierra et al., 1997, Seike et al., 2011). A co-culture of astrocytes with lung cancer brain metastasis, leads to ERK1/2 and AKT phosphorylation in cancer cells, both of which are important signaling pathways for cancer proliferation (Langley et al., 2009). Moreover, in 2015, another study showed that astrocytes presented a significant role in the downregulation of an important tumor suppressor gene, PTEN. Lin Zhang and co-workers demonstrated that in the case of breast cancer brain metastases, astrocytes release microRNAs in their exosomes that epigenetically regulate the expression of PTEN. Interestingly, the down regulation of PTEN was only found in brain metastasis, demonstrating the importance of the microenvironment for metastasis outgrowth (Zhang et al., 2015). Although these findings support paracrine modulation, rather than a direct physical cell-to-cell interaction, the later should not be ruled out.

Along with cell proliferation, migration and invasion are also important features of brain cancer, and are extremely relevant in the outcome of the disease. Several studies already showed that astrocytes are able to promote invasion and migration of glioma cell lines, and of other cancer cell lines (Placone et al., 2016). In 2003, a study using a glioblastoma cell line (U251), revealed that this cell line displays an increased invasion capacity when co-cultured with astrocytes. This effect was attributed to the activation of the inactive pro-MMP2 released by astrocytes. The active form of the MMP -2 is able to degrade collagen IV, one of the major components of ECM, facilitating the infiltration and invasion of glioma cells (Le et al., 2003). More recently, a study using ACM showed an enhancement on the invasion potential of glioblastoma stem-like cells using a trans-well invasion assay (Rath et al., 2013). One of proteins secreted by astrocytes was IL-6, which is able to promote both growth and invasion of glioma cell lines (Li et al., 2010). Moreover, in the last year, a study revealed that the hetero-cellular communication between astrocytes and glioma cells, namely through the gap junctions formed between them, are related with the invasion capacity of glioma cells. In fact, when a transgenic mice without Cx43 in astrocytes was used, perturbing the formation of hetero-cellular channels between glioma cells and astrocytes, a significant decrease of infiltrative edges in the tumor border was observed (Sin et al., 2016). The astrocytic effect in invasion of cancer cells also covers brain metastasis of different cancer types. In primary brain tumors, as well as in human breast and lung cell lines, matrix metalloproteinases, namely MMP-2 and MMP-9, are in part responsible for the astrocyte media-induced tumor cell invasion. In fact, the use of an inhibitor

of MMPs revealed a loss of invasive capacity of these cell lines *in vitro*, and a decreased capacity to form brain metastasis in a mice model (Wang et al., 2013). The ACM was also able to promote an increase of invasion on melanoma brain metastasis, where factors secreted by astrocytes appear to enhance the migration (Klein et al., 2015).

Another role of astrocytes in gliomas, is their involvement in the evasion of cancer cells from the immune system (Hanahan and Weinberg, 2011, Placone et al., 2016). In fact, factors released by reactive astrocytes have a range of actions that can explain this evasion to the natural killer cells and T lymphocytes, immune system cells, responsible for the elimination of non-natural cells in the human body (Placone et al., 2016). The release of molecules by astrocytes is associated with a constitutive activation of STAT-3 in glioma cells, that results in a suppressive effect in the release of pro-inflammatory cytokines, preventing the response of T-cells. In fact, a co-culture experiment of normal human astrocytes with T cells resulted in a inhibitory effector function (Gomez and Kruse, 2006, Kostianovsky et al., 2008a, O'Brien et al., 2013). Moreover, reactive astrocytes are able to release several other immunomodulatory cytokines, namely IL-10. Among its several functions, IL-10 appears to be responsible for a reduction of antigen presentation, through a down-regulation of monocyte MHC class II expression, as well as for an inhibition of T-cell activity, which protects the neoplastic cells in a cancer condition (Grutz, 2005). Through downregulating of tumor necrosis factor alpha (TNF-α) expression, astrocytes stop the up-regulation of MHC II in microglia and macrophages leading to an impairment in the presentation of antigens to T-cells (Kostianovsky et al., 2008b). The immune protection of cancer cells provided by astrocytes, targets mostly the usual functions of T-cells as their activation or recruitment. Moreover the ECM glycoprotein Tenascin-C (TNC), released by astrocytes, is responsible to mediate cell-cell and cell-matrix interactions. The role of ECM has been demonstrated as crucial for cancer, not only by the capacity of TNC to promote glioma invasion, but also to inhibit the transmigration through tumor monolayer by T-cells (Huang et al., 2010, Xia et al., 2016).

Astrocytes, have also been shown to directly protect glioma cells against different drugs, currently used in GBM therapeutics. For instance, the assessment of apoptosis index in glioma cells revealed a decrease in cell death superior to 50% upon treatment with TMZ, when the gliomas are co-cultured with astrocytes. Interestingly, this effect was lost when the researchers try to use ACM or a gap junction inhibitor suggesting that the chemo-protective effect requires a direct contact between astrocytes and glioma cells through connexin 43-based gap junctions (Chen et al., 2015). The same protective effect was also observed when cell lines of brain metastases were exposed to the chemotherapeutic agent paclitaxel (Taxol). The reason for this protective effect is not well understood, but processes such as the

uptake and retention of Ca^{2+} by astrocytes, or even small non coding RNAs (sRNAs) released by astrocytes, have already been associated to this effect in brain metastases (Lin et al., 2010, Menachem et al., 2016)

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In sum, these studies attribute to astrocytes putative roles in the pathophysiology of primary brain tumors and brain metastases(Figure 1.5). However, the majority of these conclusions were obtained from *in vitro* studies, with obvious limitations. Taking in consideration the different cell types present in the brain and the interactions between them in the context of brain tumors, these studies fail to simulate the microenvironment complexity around the tumor. Considering that astrocytes communicate with brain cancer cells, it is crucial to understand the impact that these cells may have on glioma, and how the interaction with the surrounding cells in the tumor microenvironment *in vivo* can modulate disease progression.

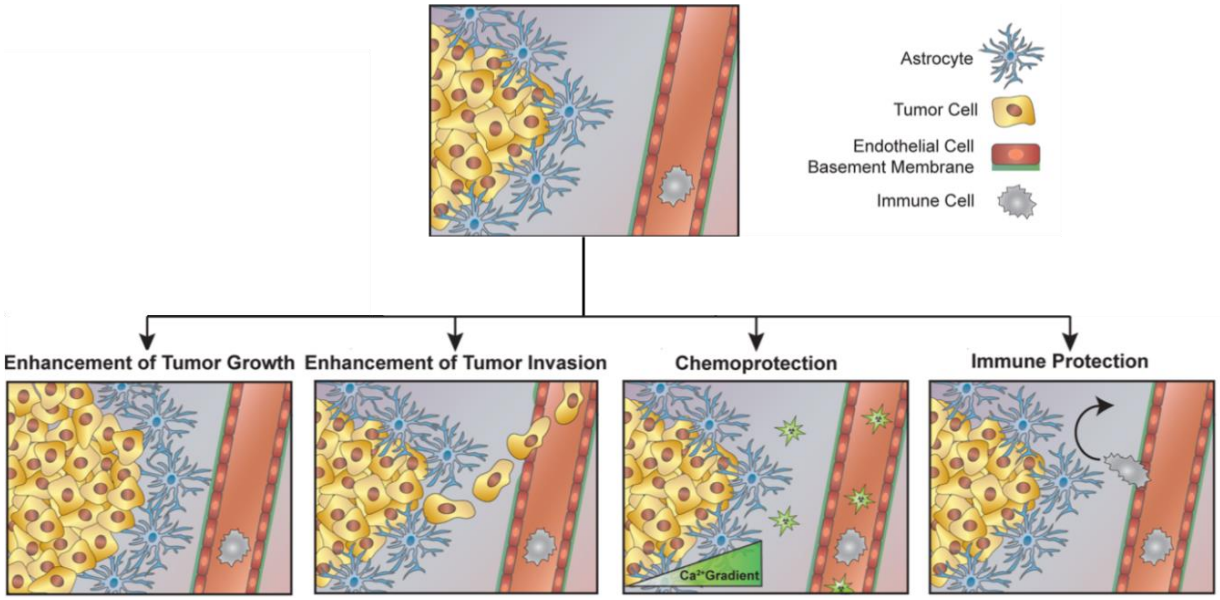


Figure 1.5 - Putative roles of astrocytes in cancer progression - Astrocytes may modulate cancer progression through the enhancement of tumor growth and invasion, chemoprotection, and immune escape. Several secreted products by astrocytes can enhance cancer progression by acting directly with neoplastic cells, or by interfering with the role of other non-neoplastic cells (*e.g.* microglia, macrophages, lymphocytes). Astrocytes can also modulate the extracellular matrix (ECM) conformation, presenting a direct implication in the invasion of cancer cells (Adapted from (Placone et al., 2016)).

1.4 The dnSNARE model for study of astrocyte modulation of microenvironment

In order to assess the role of astrocytic vesicular release in GBM modulation *in vivo*, it was necessary to select a model with a specific impairment on this capacity. Several models in which astrocytes are modulated by genetic modifications, have been developed to understand the relevance of astrocytes in normal and diseased brain (Oliveira et al., 2015). Among them, the models in which the astrocytes are not able to release substances by exocytosis, such as the dnSNARE, Glast-iBot and GFAP-TeNT, appear as good candidates for the study of astrocytes influence in GBM. The dnSNARE model was selected, since the dnSNARE expression in astrocytes was showed by independent studies to impact the release of gliotransmitters in cell culture conditions and *in vivo* dnSNARE mice (Zhang et al., 2004, Pascual et al., 2005, Sultan et al., 2015) Moreover, being an inducible model, it prevents developmental effects that could mask potential alterations in the brain microenvironment. The dnSNARE model has been selected for studies in different disorders, among which stand out the studies regarding sleep deprivation and the subsequent effect on cognitive/emotional processes (Halassa et al., 2009, Florian et al., 2011). The release of ATP by astrocytes presented an active role for sleep homeostasis. Moreover, studies with this transgenic model suggested that astrocytes were able to modulate epileptogenesis and pathophysiological consequences of epilepsy through pathways involving N-methyl-D-aspartate (NMDA) receptors (Clasadonte et al., 2013). The targeting of astrocytes signaling, mediated by the vesicular release, have been suggested to have a potential benefit for the outcome of stroke in human patients by limiting the spread of damage (Hines and Haydon, 2013). In conclusion, this model was validated by different laboratories and is highly suitable for the purpose of this project.

1.5 Research goals

GBM are the most common and lethal tumors of the CNS, the median survival for the patients is approximately 15 months, with the present treatment. The current treatments available that target almost indiscriminately neoplastic and non-neoplastic cells have been insufficient against GBM. The role and importance of the glioblastoma microenvironment has been emphaticized in recent years, and future therapies may use the microenvironment as an additional target. Astrocytes constitute the majority of glial cells in the brain, and have been described to have an active role in the pathophysiology of GBM. Although the mechanisms are still unclear, *in vitro* studies, have revealed the importance of paracrine regulation of glioblastoma cells by astrocytes. In this work, it is hypothesized that the secreted products released in vesicles by astrocytes can be responsible for an influence in glioblastoma growth and invasion. To clarify this hypothesis, the dnSNARE mouse model that displays with an impairment in astrocytic vesicular release was studied. In this model, astrocytes have an impaired capacity to modulate the ECM via astrocytic vesicular release. Therefore, *in vitro* and *in vivo* complementary approaches were employed to assess whether the astrocytic modulation of the ECM via exocytosis influences GBM. We aimed to evaluate:

1) The effect of conditioned medium secreted by glial cultures derived from WT and dnSNARE mice in viability and migration capacity of a glioma cell line;
2) The effect of astrocytic vesicular release in tumor growth and mice survival, using an *in vivo* syngeneic orthotopic intracranial GBM model;

Chapter 2 – Materials and Methods

2 Materials and Methods

2.1 Cell lines and culture conditions

The mouse glioma 261 cell line (GL261), a kindly donation of Prof. Conceição Pedroso Lima from Center for Neuroscience and Cell Biology, was used in this project. In 1939, Seligman and Shear, obtained a carcinogenic induced mouse glioma model (GL261) through intracranial implantation of methylcholanthrene pellets in the brain mice, and since then this glioma model has been used in the study of GBM (Seligman et al., 1939, Newcomb and Zagzag, 2009).

Cells were cultured in Dulbecco´s Modified Eagle Medium (DMEM; Gibco®, USA) supplemented with 10% Fetal Bovine Serum (FBS; Biochrom, UK) and 1% Penicillin-Streptomycin (Invitrogen, USA), which will be designated as complete DMEM from now on. Cells were maintained in a humidified atmosphere at 37°C and 5% (v/v) CO_2, and passaged to new flasks at sub-confluent levels. To perform *in vitro* assays, when 80% confluence was reached, GL261 cells were washed with PBS and detached with trypsin at 37°C for 5 minutes. Trypsin was inactivated using complete DMEM (twice the trypsin volume), collected and centrifuged at 900 rpm for 5 minutes. Cells were resuspended in complete DMEM, and using a 1:1 dilution of trypan blue dye and cell suspension, cells were counted using a Neubauer chamber. Cell density was calculated accordingly with the different assays performed.

2.2 The dnSNARE mouse model

Experiments were conducted in mice expressing a transgenic dominant-negative domain of vesicular SNARE (dnSNARE) and their respective Wild-Type (WT) littermates were used as controls. Animals were obtained using crossing two transgenic mouse lines: GFAP-tTA, in which the expression of tetracycline transactivator (tTA) is mediated by the GFAP promoter; tetO.dnSNARE, in which the dominant-negative domain of vesicular SNARE (Synaptobrevin II/ VAMP2), the reporter enhanced green fluorescent protein (EGFP) and *lac*Z domain are coexpressed under the control of the tetO promoter. The dnSNARE mice present a "Tet-Off" tetracycline transcriptional activation system where in the absence of doxycycline (dox) the tTA protein binds to tetO operator, triggering the transgene expression, and blocking the vesicle fusion (v-SNARE) with plasma membrane domain (t-SNARE; Figure 2.1). The impairment in SNARE

complex assembly driven by the GFAP promoter results in blockade of exocytosis specifically in GFAP+ cells (Pascual et al., 2005, Fujita et al., 2014, Sultan et al., 2015).

To prevent expression of dnSNARE during mice development, doxycycline (Sigma-Aldrich) was administered in the drinking water (100 µg/ml), and removed 4 weeks before the experiments. Animals were kept in facilities with 12 h light/dark cycle with *ad libitum* access to food and water.

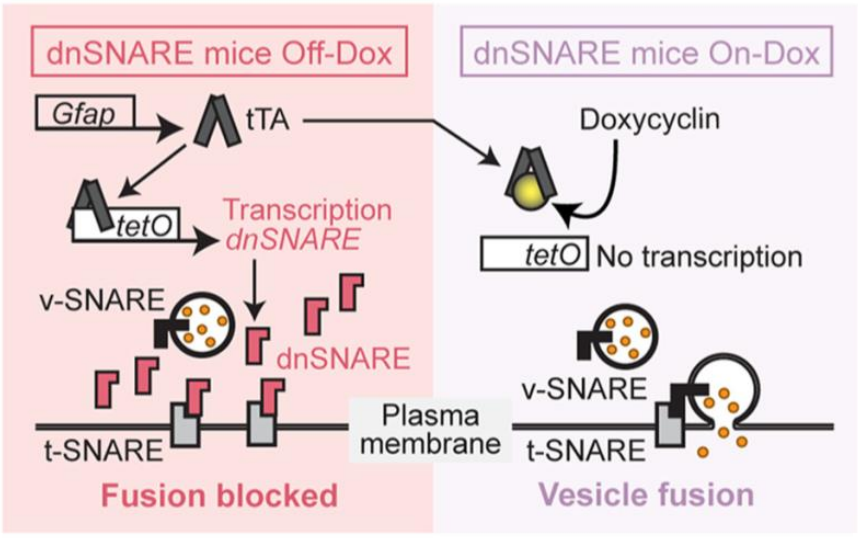


Figure 2.1. dnSNARE model – Schematic outlining of the "Tet-Off" system used in the transgenic model to impair exocytosis specifically in astrocytes (Fujita et al., 2014).

2.3 Mouse genotyping

Mice were ear tagged using a scissor, and a tail sample was collected for genotyping proposes. In ice, 300 µl of Cell Lysis (Citomed, Portugal) and 1.5 µl of proteinase K (200 mg/mL; Citomed, Portugal) were added to each sample, followed by a spin down to collect the supernatant. The samples were then left overnight to allow the tissue dissociation. In the next day, 100 µl of Protein Precipitation solution (Citomed, Portugal) were added to the cell lysis and after a quick vortex for homogenization, the samples were centrifuged at 14000 rpm during 5 minutes. To induce DNA precipitation, 300 µl of Isopropanol (100 %) were added to the samples, followed by a centrifugation (14000 rpm; 5min). The supernatant was carefully discarded and 300 µl of Ethanol 70 % were added to the pellet. One more centrifugation was done (14000 rpm; 1 min), the supernatant removed and the pellet was left to dry at

room temperature for 60 minutes. Finally, miliQ water was added to the samples and left to incubate at 65°C for 1 h.

The genotyping was carried out by the polymerase chain reaction (PCR) technique with two pairs of primers, tTA and tetO, that were used in separated PCR mixtures to identify the transgenic mice. Additionally, the constitutive gene HSF-1 was used for control effects. The PCRs were performed in a thermocycler (Mastercycler®, Eppendorf, USA), and the amplified PCR products were separated on a 1.2% agarose gel prepared in Tris-Acetate-EDTA (TAE) running buffer that was boil, before the addition of the green safe (2%). DNA size marker and the samples were loaded in the gel, and electrophoresis at 150V run for 1h. Gel pictures were taken using a transilluminator (Alpha Innotech Corporation, Bio-Rad). The primer sequences and PCR conditions, used for genotyping are present in Table 1.

Table 1 - PCR conditions for genotype identification

Primer sequences for PCR	
Primer	**Sequences**
HSF-1 KO1	5´ - TCT CCT GTC CTG TGT GCC TAG C – 3´
HSF-1 KO2	5´- CAG GTC AAC TGC CTA CAC AGA CC – 3´
tTa forward	5´ - ACT CAG CGC TGT GGG GCA TT – 3´
tTa reverse	5´ - GGC TGT ACG CGG ACC CAC TT – 3´
TSL forward	5´- TGG ATA AAG AAG CTC ATT AAT TGT CA – 3
TSL reverse	5´- GCG GAT CCA GCA ATG ATA AGA – 3´

Reaction mix components (10 µl/reaction)			
Mix/sample (µl) for tTa		**Mix/sample (µl) for tetO**	
Buffer (NH4) SO4 10X	1	Buffer (NH4) SO4 10X	1
MgCl2 (25mM)	1.2	MgCl2 (25mM)	1.2
DMSO 99.9%	0.24	DMSO 99.9%	0.24
dNTPs (10mM)	0.24	dNTPs (10mM)	0.24
Primer tTa forward	0.4	Primer TSL forward	0.6
Primer tTa reverse	0.4	Primer TSL reverse	0.6
Primer HSF-1 KO2	0.3	Primer HSF-1 KO2	0.3
Primer HSF-1 KO2	0.3	Primer HSF-1 KO2	0.3
Taq DNA Polymerase	0.4	Taq DNA Polymerase	0.4
H20 miliQ	5.52	H20 miliQ	5.12

Amplification program (40 cycles)		
Step	**Temperature (°C)**	**Duration (sec)**
Initial Denaturation	94	300
Denaturation	94	60
Annealing	61.6	60
Extension	72	60
Final extension	72	600

2.4 Primary culture of glial cells

Primary cultures of glial cells were obtained using a modified protocol of Schildge and colleagues (Schildge et al., 2013). Mice between 5 and 7 days' age (P5-P7) previously genotyped were sacrificed by decapitation and the brain was removed and placed in a cold Hank´s Balanced Salt Solution (HBSS), to maintain pH and osmotic balance of the tissue. Using a magnifier, the olfactory bulbs and brainstem were removed and the hemispheres were separated and open. The hippocampus was carefully removed from both hemispheres, obtaining two cortexes that were used to follow the procedure. Meninges surrounding the cortical tissue were cautiously removed, to avoid contamination by meningeal cells and fibroblasts, and two "clean" cortex were obtained from each pup. Then, the samples from each animal were cut in several pieces and a quick spindown was done to pellet cortex tissue pieces. The supernatant was removed and 1 mL of dissociation medium (2.5 % trypsin; 87.5 % HBSS; 10 % DNase) was added per animal, followed by a 30 minutes' incubation of the tissue in water bath at 37°C. Next, a STOP solution (40% FBS and 60% HBSS) was added to inactivate trypsin, followed by a centrifugation at 800 rpm for 2 minutes. After centrifugation the supernatant was discarded and 1.5 mL of complete DMEM was added per sample. The samples were then vigorously resuspended with a pipette until no brain tissue was visible at human eye. One more centrifugation was done and the medium was changed, followed by a new resuspension. At last, with a Neubauer chamber a sample of cell suspension plus Trypan Blue was added (1:1) for quantification effects. For each T25 cell culture flask, 7 x 10^5 cells were placed, and the medium (complete DMEM) was replaced after the first 48 hours, followed by a medium renovation each 3 days. The primary cells were left to grow for 18-20 days in an atmosphere at 37°C supplement with 5% (v/v) CO2, the time necessary to reach an 80-90 % confluent state.

To obtain conditioned medium (CM) of glial cultures, cells in culture for 18-20 days were washed twice with PBS followed by another wash with DMEM without FBS. A volume of 3 mL of DMEM (1% Pen-Strep) per T25 was added, and glial cultures were placed at an incubator at 37°C for 24 hours. After this time, CM derived from WT glial cultures (CM WT) and from dnSNARE glial cultures (CM dnSNARE), were collected and filtered (0.2 µm), followed by a "snap freeze" in liquid nitrogen for 5 minutes. Samples were stored at 80°C negative, until needed.

2.5 Viability assays

2.5.1 Trypan blue assay

Trypan blue assay is a well described test use to determine the number of viable cells present in a cell suspension. The principle is based that viable cells have an intact cell membrane and are able to exclude the trypan blue dye, where dead cells do not, presenting thus a blue cytoplasm (Strober, 2015). GL261 cells were plated in 12-well cell plates at a density of 2 x 10^4 cells per well and allowed to adhere and grow in a complete DMEM for 48 hours. After this period, cells were washed twice with PBS, and conditioned medium (CM WT or CM dnSNARE) were added to the cells. For control effects, GL261 were also grown in to DMEM with 0 % FBS and DMEM with 10 % FBS. After 48 hours of CM exposure, cells were washed with 500 µl of PBS, followed with 200 µl of trypsin during 5 minutes at 37°C. Trypsin was inactivated with 200 µl of complete DMEM, and 20 µl of cell suspension were collected to eppendorfs to which was added 20 µl of trypan blue solution. From this mix, 10 µl were placed in a Neubauer Chamber for viable cells counting effects. The results represent the mean of at least three independent experiments, each one in duplicate, and are normalized for the control group (0 % FBS).

2.5.2 MTT assay

MTT (3-(4, 5, - dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) tetrazolium reduction assay is a well stablished technique to analyze cell viability and metabolic cell activity. Viable cells are able to cleavage the tetrazolium salt MTT into formazan (blue/purple colored product), by the mitochondrial enzyme succinate-dehydrogenase. The quantity of formazan produced, absorbance recorded at 570 nm, is proportional to the number of metabolic active cells present in the sample (Slater et al., 1963, Denizot and Lang, 1986). GL261 cells were plated in 24-well cell plates at a density of

1 x 10^4 cells per well, and left to adhere and grow in a complete DMEM medium for 48 hours. Then, conditioned medium (CM WT and CM dnSNARE) were added to the attached cells for additional 48 hours. For control effects, GL261 were also grown in DMEM with 0 % or 10 % FBS. A solution of MTT (0.5mg per 1mL of PBS; Life Technologies, USA) was prepared, and 300 µl of the solution were added per well, followed by an incubation in a humidified atmosphere, at 37°C and 5% (v/v) CO2, for 1 hour. MTT was then removed and 300 µl/well of acid-isopropanol (0.4 M of HCl in isopropanol) was added to dissolve the purple crystals. Finally, the absorbance at 570 nm was measured using a plate reading spectrophotometer (ThermoFisher scientific, USA). The results represent the mean of at least three independent experiments, each one in duplicate, and are normalized for the control group (0 % FBS).

2.6 Migration assay

To understand how astrocytes can contribute for the migration of glioblastoma cells *in vitro*, a Wound-Healing Assay was performed in GL261 cells exposed to CMs from WT or dnSNARE glial cultures. The Wound-Healing Assay, is based on observation of directional cell migration into a "wound" created on a cell monolayer. The assay presents a reliable way to studied directional cell migration, that is mainly regulated by the ECM-cell interaction and soluble factors presents in the medium (Rodriguez et al., 2005).

GL261 cell were seeded in 12-well cell plate at a concentration of 1 x 10^5 cells/well, and left to growth in a humidified atmosphere, at 37°C and 5 % (v/v) CO_2, until a monolayer was formed. The "wound" was made by manual scratching with a 200 µl pipette tip, and CM from both genotypes and controls, were added to the cells. At this point, the "wounded" areas were photographed in 6 distinct places (time point: 0h), at 10x magnification using an Olympus IX51 inverted microscope equipped with an Olympus DP20 Digital Camera System, for quantification effects. The "wound" areas were photographed in the same 6 places, after 24, 48 and 72 hours of exposition to CM. The results are present as percentage of wound closure, and were calculated using the b-wound software. The distance of the wounds was measured in 10 distinct points per photo, and presented in raw pixels. The mean wound distance was calculated per condition in the 4 time points (0, 24, 48, and 72 hours). Wound closure (%) was calculated by dividing the wound distance at 24,48 and 72 hours, per the wound distance at 0h. The results represent the mean of at least three independent experiments, each one in duplicate, and are normalized for the control group (0 % FBS).

2.7 Intracranial orthotopic glioma models

To understand the impact of the astrocytic vesicular release in glioma growth and progression an *in vivo* approach was used. The transplantation of murine syngeneic glioma cells into mice, is the best available approach when the tumor microenvironment is being targeted. The use of this model allows to mimic closely the interaction between the tumor and non-tumor cells (*e.g.* immune system cells) mimicking better the inter-cellular interactions also present in the human disease (Newcomb and Zagzag, 2009).

For the establishment of GBM model, GL261 glioma cells were prepared at a concentration of 5×10^4 per 5 µl. The mice were anesthetized by intraperitoneal injection with a solution composed by ketamine (75 mg/kg, Imalgene 1000, Merial, USA) and medetomidine (1 mg/kg, Dorbene vet, Loetis, Spain). Mice were placed in a stereotaxic apparatus, where the dorsal head surface was disinfected with 70% alcohol, and using a scalpel a small incision was done until the scalp was reached. The exposed area of the scalp was clean and dried, and a hole was made with a small drill at 1.8 mm medial-lateral right and 0.4 mm anterior-posterior from bregma. At this point, mice received a stereotactic injection of 5×10^4 GL261 cells resuspended in 5 µl of sterile PBS, using a Hamilton syringe. The needle of the syringe was inserted 2.5 mm below the brain surface, and cells were slowly injected (5 µl per minute) at a volume of 1 µl of cell suspension each 0.5 mm depth in the brain, creating a column of GL261 cells in the mice brain. After the procedure, animals were sutured with 0.2 µm suture line, and atipamezol (1mg/kg, AntiSedan, Orion, Finland) was subcutaneously administrated for anesthesia recover. During this time, animals were placed under a heat lamp, and when totally awake, transferred for their respective cages, where they were monitored daily.

2.7.1 Survival study

To determine the survival of WT (n=23) and dnSNARE (n=12) mice were weighted every three days, and sacrificed when they presented moribund symptoms or body weight loss higher than 30%. Brain tissues were collected for immunohistochemistry analyses, on which samples were fixed by immersion in 4 % paraformaldehyde (PFA) and subsequently embedded in paraffin.

2.7.2 Tumor volume assessment

For tumor volume measures, WT (n = 14) and dnSNARE (n = 15) were sacrificed 14 days after intracranial injection with 5 x 10^4 GL261 cells. Brain tissues were collected for immunohistochemical analyses, in which samples were fixed by immersion in PFA 4 % and subsequently embedded in paraffin. Samples were then cut using in a vibratome, in slices of 4 µm thickness in intervals of 100 µm. The slices were stained with Hematoxylin and Eosin (H&E), for nuclear and cytoplasmic coloration allowing histological characterization. Tumor cells, presenting an aberrant morphology with pleomorphic nucleus, were distinguishable in H&E staining. Using the AxioVision SE64 Rel.4.9.1 software (Carl Zeiss, Germany) the tumor area was measured in each slice, and the volume was calculated multiplying the area of tumor in each slice for 100 µm (distance between slices) to estimate total tumor volume across the several sections.

2.8 Histological analyses

2.8.1 Immunofluorescence

Glial Cultures

At the end of glial cultures isolation procedure, 5 x 10^4 cells were placed per Poly-D coated lamella. Immunofluorescence of glial cultures was performed 20 days after the establishment of the primary cultures. Glial cells were fixed using 500 µL per well of PFA (4%) for 30 minutes at room temperature (RT). After fixation, cells were washed three times with PBS followed by cells permeabilization with 0.3 % v/v Triton-X 100 in PBS for 5 minutes. Cells were then washed three additional times, and PBS with 10% FBS was added during 1 hour at RT to reduce unspecific ligations. Cells were washed one more time, and lamellas with cells were placed in humid chamber and covered with the primary antibody diluted in PBS with 10 % FBS for 1 hour at RT After this time the secondary antibody was added, to the cells previously washed, for 1 additional hour. To finalize the procedure, DAPI (1:1000; Invitrogen, USA) was added for 5 minutes at RT, followed by a final wash with PBS. Finally, lamellas were mounded using Immuno-Mount (ThermoFisher Scientific, USA) and stored at 4°C. The specifications of antibodies used are listed in Table 2.

Brain Samples

Brain tissue sections were deparaffinized and rehydrated by xylene and ethanol series. Samples were then washed with PBS, and permebialized for 10 minutes in a PBS-T (0.3%) solution. After new wash, a citrate buffer solution (Sigma-Aldrich, USA) was used for antigen retrieval, being the samples submerged in hot citrate for 20 minutes. Samples were washed one more time and PBS with 10 % FBS was added, to reduce unspecific bounds, for 30 minutes at RT. The samples were incubated overnight with primary antibodies diluted in PBS-T 0.3 % with 4 % FBS, in a humid chamber. In the following day, the samples were washed and incubated with the respective secondary antibodies diluted in PBS and 4 % FBS, for two hours at RT. In the dark, samples were washed again and incubated at RT for 10 minutes with DAPI (1:1000; Invitrogen, USA). The protocol ended with a new wash, and the mounting of the samples using Immu-mount (ThermoFisher Scientific, USA) and stored at 4°C. For comparative effects, the immunofluorescence against GFP was performed in 6 brain sections per animal in the same conditions. Photos used to quantify immunofluorescence were acquired in the same conditions. GFP expression was analyzed using Fiji open source software (http://fiji.sc/Fiji) by measuring the mean gray value per animal, representative of the fluorescence intensity. The list of antibodies used is present in Table 2.

Table 2 - Information about the antibodies used for immunofluorescence

Primary Antibodies	Specie	Dilution	Company
anti-GFAP	Rabbit	1:200	DakoCytomation, Denmark
anti-GFP	Goat	1:300	Abcam, UK
Secondary Antibodies	**Specie**	**Dilution**	**Company**
Alexa Fluor® 594 anti-rabbit	Donkey	1:1000	ThermoFisher Scientific, USA
Alexa Fluor® 488 anti-goat	Donkey	1:1000	ThermoFisher Scientific, USA

2.8.2 Western blot

The Western Blot (also named protein immunoblot) is a technique that allows the identification and separation of proteins according to their size by gel electrophoresis. The proteins are posteriorly transferred to a membrane and are detected by using specific antibodies (Jensen, 2012). Western Blot was performed in samples of brain tissues and in primary glial cultures, to identify and quantify proteins of interest. Brain samples were lysed in order to release the proteins of interest using a lysis buffer

containing: cold HEPES-buffered sucrose (0.32 M sucrose, 4 mM HEPES pH 7.4) with 25 X protease inhibitors, 1 % Nonidet-P40 and 0.5 % sodium dodecyl sulfate (SDS). In the case of primary glial culture samples, a lysis buffer containing: 50mM tris; 150mM NaCl; 2 mM EDTA and 1 % Nonidet-P40, was used.

Both sample types were sonicated, and for brain tissue a centrifugation at 10000 rpm (25 minutes, 4°C) took place, followed by the collection of the supernatant for new eppendorfs. To determine the total amount of protein in each sample, the Bradford protein assay (Bio-Rad, USA) was used. The method is based in a colorimetric detection that should be proportional to the protein concentration present in the sample. The protein concentration was calculated based on a standard curve obtained with several dilutions of bovine serum albumin (BSA) (0.5, 1, 3, 4, 8, 10, 16 µg/ml). Samples were measured at 595 nm in a spectrometer microplate reader (Bio-Rad, USA), and volumes corresponding to 50 µg of total protein were calculated. Total lysates were denatured in 2x Laemmli Buffer (Bio-Rad, USA) by heating the samples at 98°C for 5 minutes. The next step was the SDS-Page step using an electric field allows the separation of the proteins by their molecular weight. For the "run" a 12.5 % polyacrylamide resolving gel was prepared, followed by the preparation of a 4 % polyacrylamide stacking gel, in which the sample were loaded. Electrophoresis tank was filled with a running buffer solution, and the run started at 80 V, until the proteins correctly enter the stacking gel, and then the voltage was increased to 120 V. The SDS-gel was then transferred to a nitrocellulose membrane (Trans-blot Turbo Kit, Bio-Rad, USA) using a low molecular weight (2.5 A, 25 V, 7 minutes) protocol. After protein transference, membranes were blocked for 1 hour with 5 % non-fat milk/TBS, to prevent non-specific background binding of the primary and/or secondary antibodies. The membranes were then incubated with primary antibodies overnight at 4°C with agitation. In the next morning, the membranes were washed with TBS-T, and incubated, at RT with agitation during 2 hours, with the respective secondary antibodies (Table 3). After incubation period, the membranes were washed again in TBS-T followed by the detection of the chemiluminescent signal using the Clarity Western ECL substrate kit (Bio-Rad, USA), a gel blotting imaging system (Chemidoc, Bio-Rad, USA) and the Quantity One software (Bio-Rad, USA). The quantification of the bands was done by densitometry using Image Lab software (Bio-Rad, USA.) All the samples were normalized taking into account a loading control (α-tubulin).

Table 3 - Dilutions and information about the primary and secondary antibodies used for western-blot analyses.

Primary Antibodies	Dilution	Company
Rabbit anti-GFAP	1:50000	DakoCytomation, Denmark
Goat anti-GFP	1:2000	Abcam, UK
Mouse anti-α-tubulin	1:500	DSHB, USA
Secondary Antibodies	**Dilution**	**Company**
anti-rabbit HRP	1:15000	Bio-Rad, USA
anti-goat HRP	1:5000	Bio-Rad, USA
anti-mouse HRP	1:15000	Bio-Rad, USA

2.9 TCGA data meta-analysis in glioma patients

The cancer Genome Atlas (TCGA) provides a powerful bioinformatics tool that can be used to acquire information about gene expression, copy number alterations, DNA methylation, as well as relevant clinical information. Currently, the dataset presents information regarding 572 GBM, 27 lower-grade gliomas and 10 unmatched normal samples (McLendon et al., 2008, Pojo et al., 2015).

Clinical data from each patient included in TCGA was provided by the Biospecimen Core Resources (BCRs) and contain information regarding the age at diagnosis, gender, Karnofsky performance status (KPS), overall survival (days to death or last follow up) and treatment applied to each patient.

The data is available for download through TCGA data matrix (http://tcga-data.nci.nih.gov/tcga/dataAccessMatrix.htm). Gene expression data from each patient was hybridized by the University of North Carolina, Lineberger Comprehensive Cancer Center, using Aligen G4502A 244K (McLendon et al., 2008) To prevent duplicates, the median was used when the same patient presents more than one information regarding gene expression. The *VAMP2*, synaptobrevin 3 (*VAMP3*), synaptosome - associated protein 23 and 25 (*SNAP23* and *SNAP25*), and syntaxin 1A (*STX1A*) expression values were processed. Gene expression values were categorized as high expressers for TCGA “level 3” values higher than average expression values for each gene independently. Moreover, the co-expression of the genes above was analyzed, being the high expression SNARE group, the patients with a gene expression higher than average expression in the five genes analyzed simultaneously.

2.10 Statistical analyses

GraphPad Prism 6 software (GraphPad Software Inc., USA) was used to perform statistical analyses. Results are presented throughout as mean ± s.e.m (Standard Error Mean), and statistical comparisons were calculated with a 95 % confidence interval. Parametric tests were applied, since all data sets present Gaussian distributions. An independent t-test were applied to compare the number of GFAP positive cells in primary glial cultures (WT vs dnSNARE). For cell viability, and cell migration assays a one-way analysis of variance (ANOVA) and Tukey´s post-hoc analyses to compare the three groups were used (Control, CM WT, CM dnSNARE). To compare the tumor volumes of dnSNARE and WT animals, a one-way ANOVA was used. Regarding the effect of genotype animal in survival, a Kaplan-Meier Survival curve was presented, and the differences were evaluated by univariate analysis (Log-rank test). Correlation between GFP levels and tumor volume or survival, were assessed by Pearson correlation analyses.

The effect of VAMP2, VAMP3, SNAP23, SNAP25 and Stx1A expression levels in the overall survival of GBM patients from TCGA are represented by Kaplan- Meier survival curves, and the differences were evaluated by a multivariate survival analysis (Cox model, adjusted for the follow putative prognostic factors, patient age and gender, KPS, treatment with chemotherapy and radiation). These analyses were made with SPSS 23.0 software (IBM, USA).

Chapter 3 - Results

3 Results

3.1 In vitro studies

3.1.1 Characterization of primary glial cultures

To study the role of astrocytic vesicular release in glioma cells, primary glial cultures from WT and dnSNARE mice were stablished. The dnSNARE transgene expression in cultured astrocytes have been shown to reduce the number of fusion events in 91 % resulting in a drastic impairment in astrocytic vesicular release (Sultan et al., 2015). Primary glial cultures from both genotypes were established, and 20 days after the isolation procedure, cultures from both genotypes reached a state of sub-confluence with similar cell morphology. Similar to studies that used *in vitro* hippocampal astrocytes (Sultan et al., 2015), only dnSNARE primary glial cultures presented the expression of the gene reporter (EGFP) after 20 days in culture conditions (Figure 3.1 a). To confirm the presence of astrocytes in both glial cultures, a western blot was performed from glial cells after 20 days in culture. Cultures derived from both genotypes presented the expression of astrocytic marker GFAP, confirming the presence of astrocytes (Figure 3.1 b). Taking into account that the dnSNARE transgene corresponds to the cytosolic portion of endogenous synaptobrevin II and the inexistence of functional antibodies able to discriminate between both proteins, the expression of GFP was measured by western-blot. As expected from other studies (Sultan et al., 2015), and in concordance with the fluorescence observed (Figure 3.1 a) only cultures derived from dnSNARE mice present GFP expression (Figure 3.1 b). Moreover, dnSNARE transgenes are selectively expressed in astrocytes, since EGFP co-localizes with several astrocytic markers, such as GFAP and S100β, and not with markers of other glial cell types or neuron markers (Fellin et al., 2009). The presence of GFP in our dnSNARE primary cultures, and consequently expression of dnSNARE transgene, confirmed that astrocytes from dnSNARE mice present a significant reduction in the number of fusion events and their exocytosis mediated by synaptobrevin II is significantly affected (Fellin et al., 2009, Sultan et al., 2015).

Interestingly, glial cultures obtained from different dnSNARE mice present different levels of GFP expression (Figure 3.1 b, c). Recent studies in our group, demonstrate that dnSNARE mice express different levels of GFP that directly correlate with relative expression of dnSNARE and EGFP mRNA levels (Supplementary Figure). Moreover, the expression of GFP, appears to correlate with diverse biological effects assessed at the lab. Taking in consideration that GFP levels are representative of transgene expression, only animals with higher levels of GFP expression were used in the following *in vitro* assays. Considering that dnSNARE model only present a vesicular impairment in GFAP positive cells, an

immunofluorescence against GFAP was performed in glial cultures derived from both genotypes, for GFAP positive cells quantification. Glial cultures derived from WT and dnSNARE mice present similar percentage of GFAP positive cells (Figure 3.1 d; WT, 91.4 ± 4.2 %; dnSNARE, 89.5 ± 2.1%), and no significant statistical differences were found ($t_3 = 0.471$; $p = 0.670$). These results indicate that the CM derived from glial cultures from both genotypes derived from a similar density of astrocytes, differing merely in the presence (dnSNARE) or absence (WT) of the transgene dnSNARE.

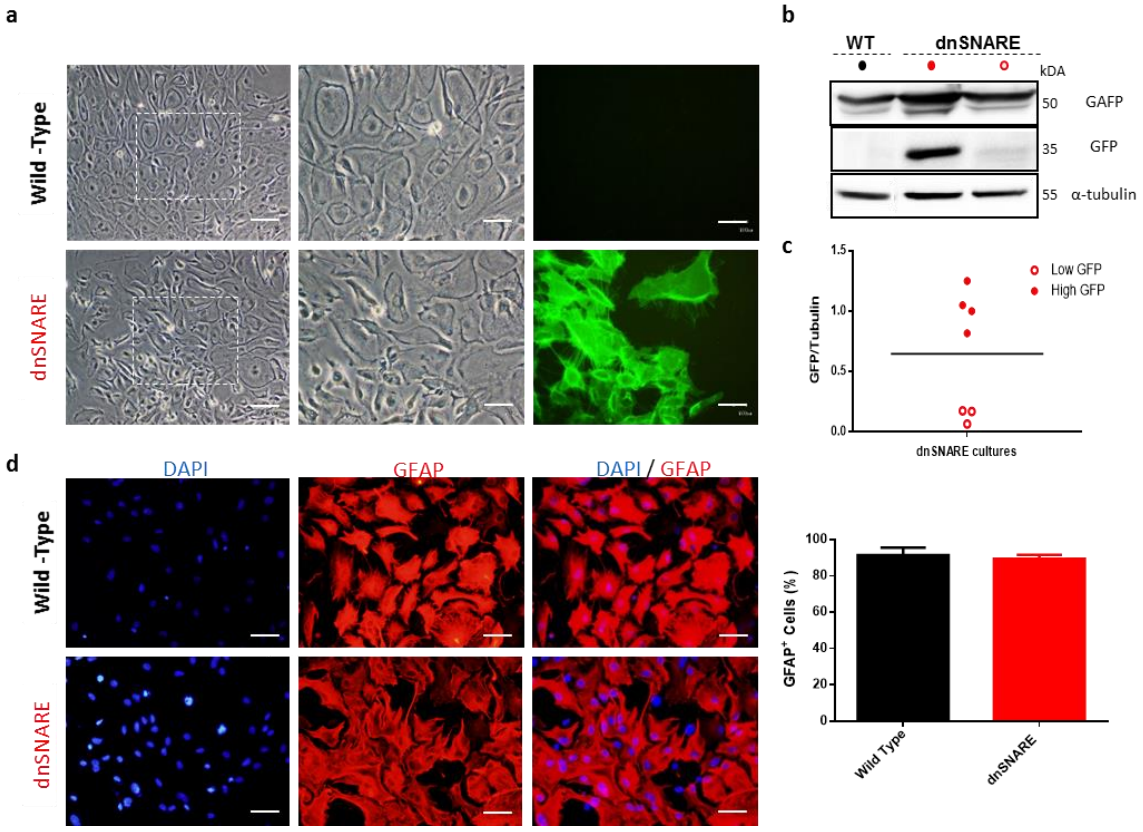


Figure 3.1 – Characterization of primary glial cultures - **(a)** Morphological characterization of glial cells after 20 days in culture (left and middle panels), and GFP fluorescence observed only in glial cultures derived from dnSNARE mice (right panels). Scale bars, (left panels: 100 µm; Middle and Right panels: 50 µm) **(b)** Western Blot against GFAP and GFP in glial cells derived from WT and dnSNARE mice. **(c)** Levels of GFP expression in glial cultures derived from different dnSNARE mice. **(d)** Immunofluorescence against GFAP in WT and dnSNARE glial cultures, and quantification of GFAP positive cells percentage. Scale bars, 100 µm. Results are presented as mean ± s.e.m.

3.1.2 Regulation of GL261 cells viability by astrocytic CM

The glial cultures established were mainly composed by astrocytes. After confirmation of the transgene expression in cultures, medium with the secreted products from astrocytes was collected. The secreted medium from glial culture, corresponding to 48 hours of secretion, was collected and applied to GL261 cells, for assessing the role of vesicular secreted astrocyte products on glioma cells viability. Glioma cells were incubated during 48 hours with CM derived from both genotypes cultures, and cell viability was assessed by the trypan blue and MTT assays.

CM derived from WT cultures lead to an increase of viable cells on glioma cells viability on the trypan blue assay comparing with the control group ($p < 0.01$). The effect was not observed when CM derived from dnSNARE cultures was added to glioma cells, suggesting a possible lack of secreted products by astrocytes. However, no significant differences were found between genotypes ($F_{2,20} = 6.085$, $p = 0.0086$; Figure 3.2 a). Similar results, were obtained when GL261 metabolic viability was assessed, by the MTT assay. The CM derived from WT glial cultures led to an increase viability on glioma cells compared with control group ($p < 0.5$), but the effect was not observed when CM derived from dnSNARE cultures was applied to glioma cells (Figure 3.2 b). Although, no statistical differences were found between the use of CM derived from the two genotypes, the secreted products by astrocytes appear to have an important role in glioma cells viability ($F_{2,26} = 4.507$; $p = 0.0209$). Based on these findings, we postulated that astrocytes might increase the viability of glioma cell by paracrine regulation, via SNARE-dependent exocytosis. The results indicate the presence of key factors in vesicles released by WT astrocytes, that are able to potentiate glioma cells viability.

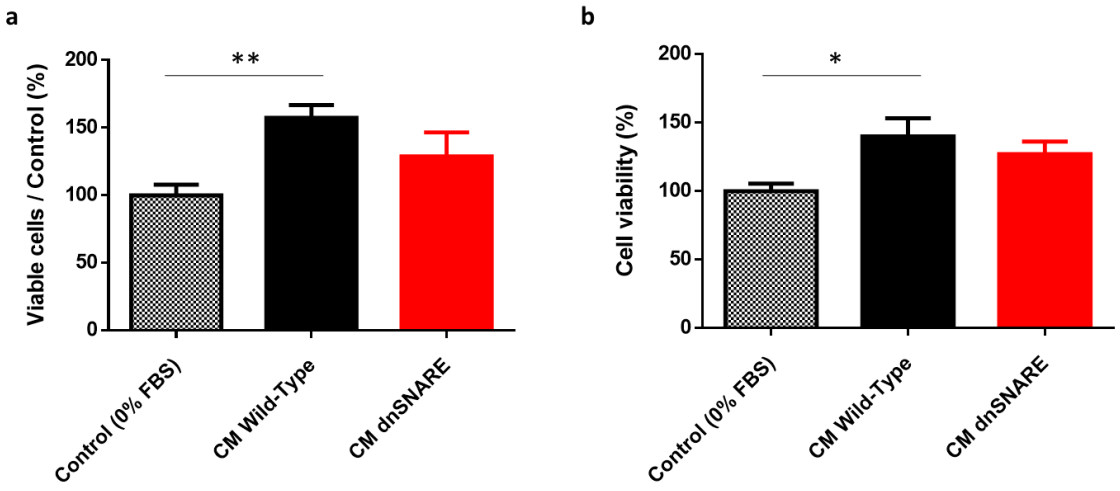


Figure 3.2 – CM derived from WT glial cultures increase glioma cells viability – Glioma cells viability measured by **(a)** Trypan Blue Assay and **(b)** MTT, after 48h exposed CM derived from WT glial cultures. **(a)** Quantification of viable cells ($p<0.01$) and **(b)** cell viability ($p<0.05$), was normalized against the control group. The results presented represent at least 3 independent assays, and are presented as mean ± s.e.m.

3.1.3 Regulation of GL261 cells migration by astrocytic CM

To study the role of astrocytic vesicular release in glioma cells, a wound healing assay was used in GL261 cells that were exposed to the CM derived from WT or dnSNARE glial cultures. The analysis of wound representative pictures (Figure 3.3 a) indicate that migration of GL261 cells was not affected by the presence of secreted substances presents in the CM. However, the migration of glioma cells was not progressive in groups tested, and the 20 % of wound closure observed after the first 24 hours was maintained after 48 and 72 hours ($F_{2, 36}=1.105$; $p = 0.3421$). The substances secreted by astrocytes did not modify the migratory profile of GL261 cells if compared with the control group at 24 hours ($F_{2, 18} = 0.1868$; $p = 0.8312$; Figure 3.3 b). However, considering that a continuous migration over time was not observed in our experiences, our results were inconclusive.

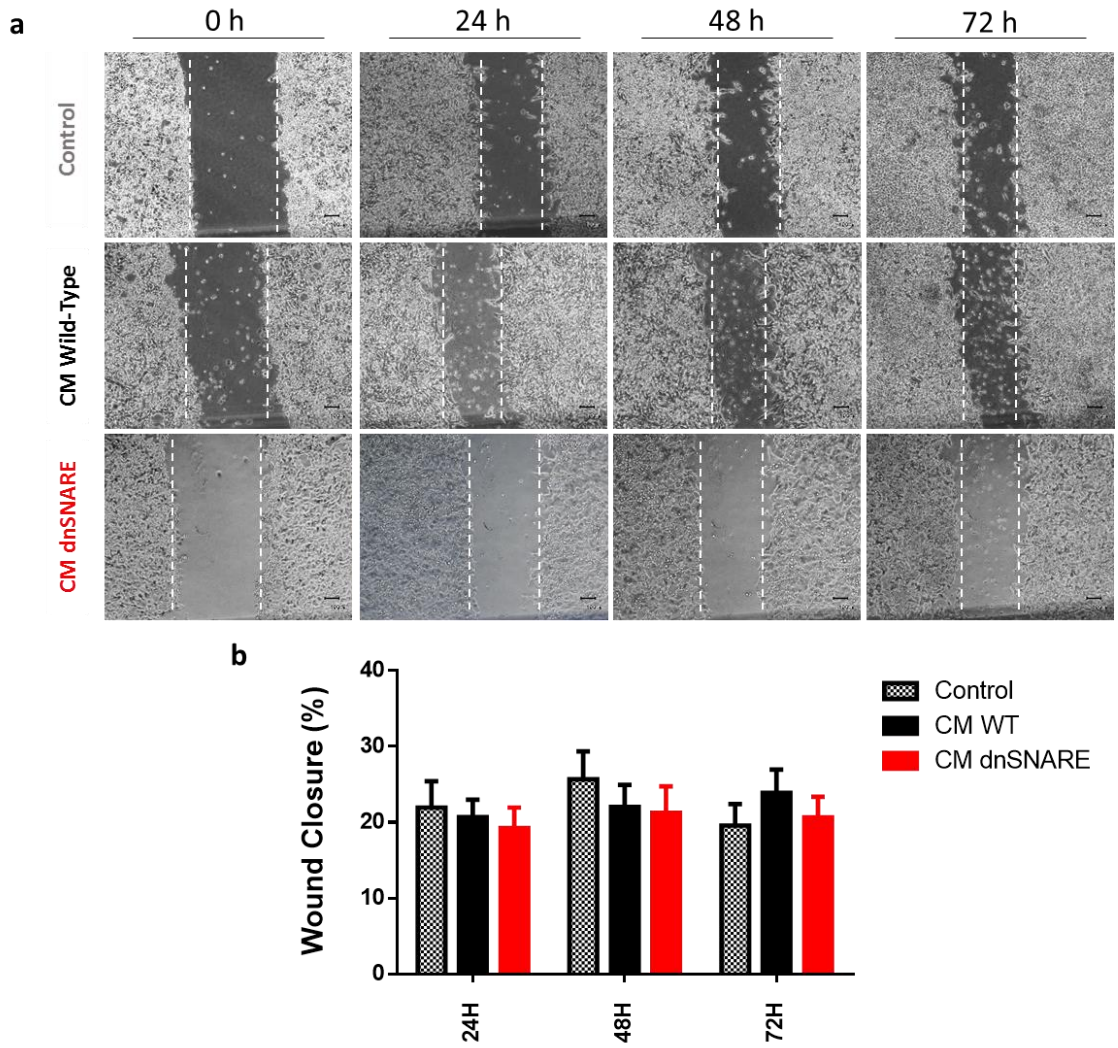


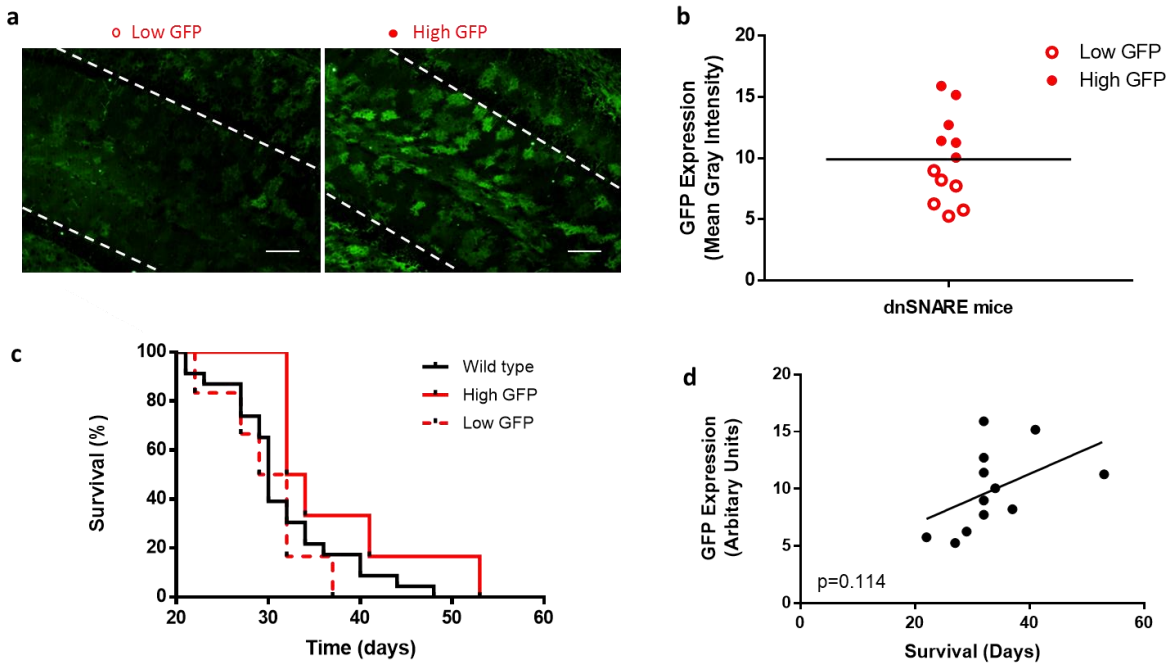
Figure 3.3 - CM derived from glial cultures do not affect glioma cells migration – Glioma cells migration measured by Wound Healing Assay. **(a)** Representative pictures of the wound at 24,48 and 72 hours in the three tested groups. Scale bars, 100 μm **(b)** Quantification of wound closure after 24, 48 and 72 hours in the three groups (control; CM WT; CM dnSNARE). The results presented represent at least 3 independent assays, and are presented as mean ± s.e.m.

3.2 In vivo studies

3.2.1 Role of astrocytic vesicular release on mice survival

Brain tumors microenvironment present a key function in the development and progression of the disease. The transgenic mouse model (dnSNARE) and respective controls (WT), were used to assess role of astrocytic vesicular release on GBM, using an orthotopic intracranial model. Mice were brain-transplanted with syngeneic GL261 glioma cells (5×10^4), and the survival of tumor-bearing mice was analyzed.

As observed in glial cultures derived from dnSNARE mice (Figure 3.1 a, b, c), and in studies at ICVS (Supplementary Figure), dnSNARE mice present different levels of GFP expression indicating distinct levels of transgene dnSNARE expression. The GFP expression was accessed by immunofluorescence in each individual (Figure 3.4 a), and dnSNARE mice were separated in 2 distinct groups considering their GFP expression: Low GFP expressers and High GFP expressers (Figure 3.4 b). Taking in a count results of our group using the same transgenic model, is expected that mice with high GFP expression, also display a higher vesicular release impairment. The 3 groups were followed and sacrificed at an *humane end point*, and survival plotted as Kaplan-Meier survival curves (Figure 3.4 c) were evaluated. Although no statistical differences were found comparing the three groups, the High GFP group tend to survive longer than WT or even Low GFP mice (High GFP vs WT, $p = 0.090$; High GFP vs Low GFP, $p= 0.066$). In accordance with these findings, are the number of animals alive in each group measured intervals of 10 days. This way, 20 days after syngeneic glioma cell injection all the animals were alive, but at day thirty only 39 % and 50 % of WT and Low GFP mice were alive against 100 % of the High GFP group. Moreover, fifty days after glioma injection only the High GFP group present animals alive (16 %) against all the mice found dead on the other groups (Figure 3.4 c). The same tendency is observed when correlated the levels of GFP presents in dnSNARE mice with their survival ($p = 0.114$; Figure 3.4 d). Despite of the lower experimental N, our results suggest that astrocytes release factors in vesicles can directly or indirectly modulate the disease progression, affecting mice survival.



Genotype	Day 20	Day 30	Day 40	Day 50
Wild Type	100 % alive	39 % alive	9 % alive	0% alive
Low GFP	100 % alive	50 % alive	0 % alive	0% alive
High GFP	100 % alive	100 % alive	33 % alive	16% alive

Figure 3.4 – Astrocytic vesicular release impairment increases survival of GBM syngeneic orthotopic model survival - (a) Representative images of GFP expression in dnSNARE mice. (Scale bars, 100 μm) **(b)** Respective GFP quantification by immunofluorescence **(c)** Kaplan Meier survival curves and percentage of alive animals at days 20, 30, 40 and 50 after glioma cells injection. **(d)** Correlation between GFP expression in dnSNARE mice and overall survival of respective mice (r = 0.4802; p =0.114).

3.2.2 Morphological assessment GBM

The brains from animals previously euthanized were collected and stored for morphological analysis. For tumor identification on brain sections of WT and dnSNARE mice, brain tissue was stained with hematoxylin and eosin. The tumor developed in animals from both genotypes present similar features at the *humane end point* and was generally located in the right subcortical brain area. At that time point, the tumor already invaded the left brain hemisphere in all mice analyzed. In mice from both genotypes, glioma cells were observed at distant localizations from the injection point, demonstrating the migration capacity of glioma cells. It was common to find tumor cells in the ventricular system, that can be use as spread pathway in the brain by the tumor cells. The H&E staining allows to delimitate the tumor mass

from the normal brain tissue, being observed specific glioma features at the tumor localization. Tumor tissue presented a high nuclear density with lack of organization, presenting prominent nuclear polymorphism and mitotic activity. The presence of necrosis was also observed in the developed tumors from both mice genotypes. These specific features only present in tumor cells create a distinguishable tumor border, where a morphological transition from normal tumor tissue to brain parenchyma was observed (Figure 3.5).

The role of astrocytes in glioma growth is yet faraway to be totally understood, but the close interaction between astrocytes and tumor cells is evident. To evaluate the disposition of GFAP positive cells, and their localization considering the tumor position, an immunofluorescence against GFAP was performed. The animals from both genotypes, presented similar GFAP positive distribution and was mainly presented around the tumor cells and in the brain parenchyma, with lower percentage of GFAP positive cells in tumor core. The higher levels of GFAP observed suggest the occurrence of reactive astrogliosis, as present in brain tumors, and a higher density of astrocytes was observed in tumor border. The increase nuclear density observed in the H&E staining was corroborated with DAPI, a common nuclear marker used in immunofluorescence. The distinguishable DAPI and GFAP staining, between tumor and brain parenchyma also created a distinguishable tumor border as observed on H&E staining (Figure 3.5).

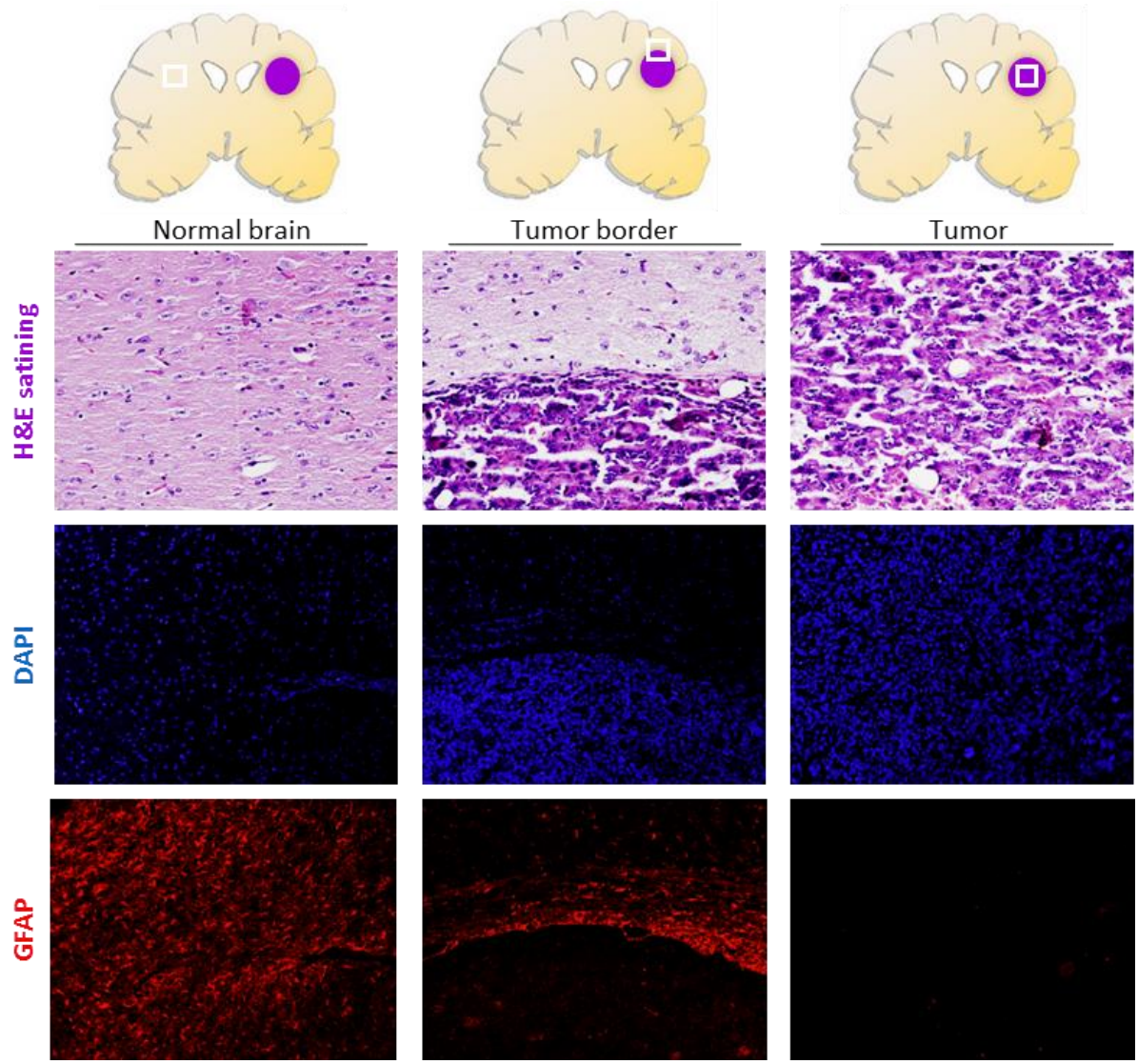


Figure 3.5 Tumor developed in dnSNARE mice present typical GBM features - Tumor formed in dnSNARE and WT animals, presenting common features of GBM. H&E stain of brain sections demonstrate the morphological differences between brain stroma and tumor tissue, creating a distinguishable border. DAPI staining reveals the difference at nuclear density observed in tumor tissue. GFAP can be found mainly expressed in the brain stroma and tumor border of the tumor, presenting a reduced expression in the tumor core.

3.2.3 Role of astrocytic vesicular release on glioma growth

Tumor growth is a major factor for the outcome of the patients with cancer brain tumors. The different rates of proliferation by cancer cells between patients lead, can be the difference of months in patient survival. The rapid and aggressive proliferation of cancer cells, is only possible in the presence of distinct factors. Between them, growth factors stimulate the cellular growth and proliferation and has been described as overexpressed in cancer cases. In the case of primary brains tumors, cancer cells are able to uptake this factors that are mainly released by astrocytes. Taking the results observed in the survival curves into account and using the same transgenic mouse model (dnSNARE), syngeneic GL261 glioma cells 5 x 10^4 glioma cells were injected intracranial and the tumor growth was accessed 14 days after injection.

Animals were injected and after 14 days was observed a significant weight loss and an abnormal posture (ex: chromodacryorrhea; back arching in some animals) in some animals, indicating the presence of brain tumors. Animals were sacrificed at this time point, and brains collected for tumor identification and tumor volume quantification. A H&E staining was performed in animals tissue from both genotypes for tumor identification and delimitation (Figure 3.6 a). It was possible to observe the presence of a tumor mass in 80 % of the tested animals, suggesting no role of genotype in tumor development. Considering that dnSNARE mice present different levels of transgene expression, as previously reported, we analyzed the GFP expression by western-blot and divided dnSNARE mice in two main groups regarding their GFP expression (Figure 3.6 b). The tumor incidence or tumor formation, after 14 days, was addressed in the three different groups (WT; Low GFP; High GFP), presenting similar results regarding the percentage of animals that developed tumor at this time point (Figure 3.6 c). Brain sections with a distant between them of 100 µm were stained with hematoxylin and eosin, for tumor identification. The tumor volume was evaluated by delimitating the main tumor mass, easily distinguishable from brain parenchyma, and multiplying the area of tumor in each section by the sum of all the sections presenting tumor. The tumor volume for the different groups was calculated and no significant differences were found ($F_{2,20} = 0.9158$; $p = 0.4163$) indicating that astrocytic vesicular release did not present a direct impact in tumor growth (Figure 3.6 d). Regarding the correlation between of tumor volumes and different levels of expression in dnSNARE mice our data failed to link these two variables (Figure 3.6 e), supporting the idea that astrocyte-derived molecules shall not interfere with the tumor size. The results presented suggest, that the tendency observed in animal survival (Figure 3.4 c) , is not related with the tumor growth but with other factors that directly influence the disease outcome.

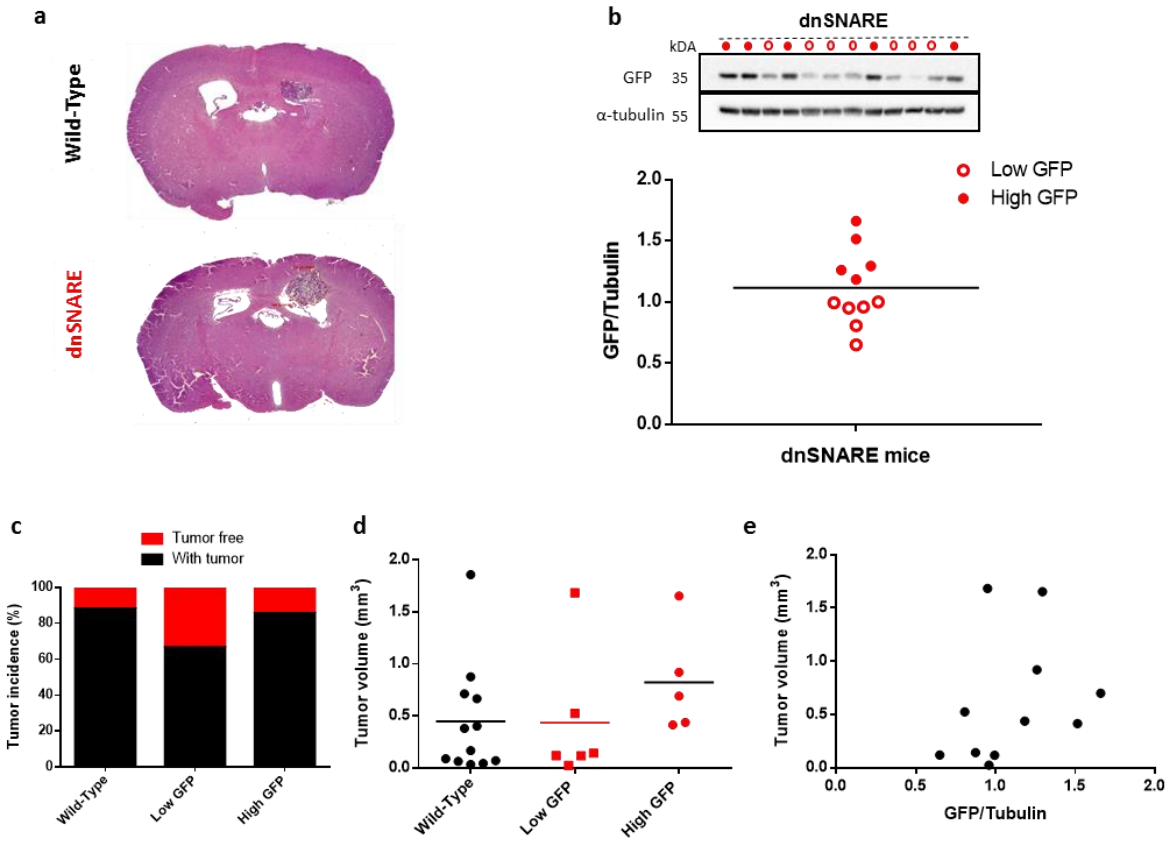


Figure 3.6 Astrocytic vesicular release impairment does not affect *in vivo* GBM growth – (a) Representative images of H&E staining, of glioma bearing mice (WT and dnSNARE). **(b)** Analyses of GFP expression in dnSNARE mice by western-blot. Representative image of the experiment results and respective quantification. Data presented was normalized for α-tubulin expression. **(c)** Tumor incidence after 14 days in tested groups. **(d)** Quantification of tumor volume and **(e)** correlation with GFP expression levels in dnSNARE mice ($p = 0.382$; $r = 0.293$).

3.3 In silico studies

3.3.1 SNARE proteins expression in GBM patients

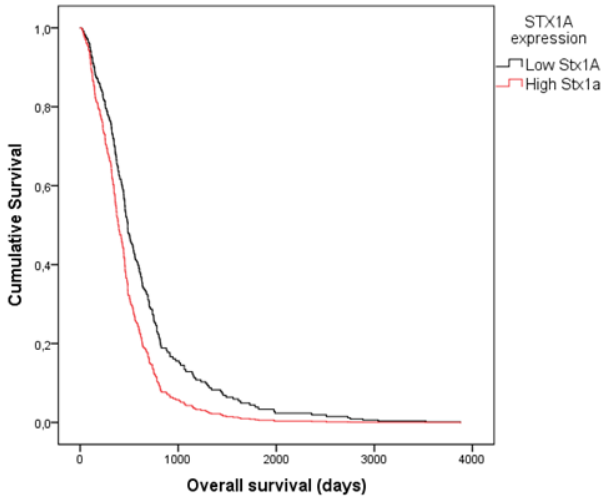
In the dnSNARE model the VAMP2 protein is manipulated resulting in a dysregulation of the SNARE complex and consequently an impairment on astrocytic vesicular release. The target of a single protein of SNARE complex, VAMP2, leads to a dysregulation of astrocytic exocytosis. Considering that SNARE complex is present in several cell types, including glioma cells and astrocytes, we evaluate if a distinct expression of SNARE complex genes (*VAMP2; VAMP3; SNAP 23; SNAP 25; STX1A*) could present

relevance in the OS of GBM patients. The expression levels of the genes previously mentioned were analyzed in 389 GBM patients deposited in TCGA.

Patients with high expression levels of *STX1A* (median OS = 372 days; 95 % CI = 332 - 411 days), presented a shorter OS when compared with patients with low expression levels of STX1A (median OS = 454 days; 95 % CI = 420 - 48 days; Figure 3.7 a). Using a multivariate survival analysis, with cox regression model, was observed an association between high levels of *STX1A* and a shorter OS of patients (Cox model p-value < 0.001), independently of other putative prognostic variables, including gender, chemotherapy, radiotherapy, age and KPS. The multivariate analyses confirmed that age, gender, KPS and radiotherapy present statistical important in the OS survival of GBM patients (Figure 3.7 b). Interestingly, chemotherapy did not present a significant cox model p-value, probably because of the discrepancy between the number of patients with and without the treatment.

The expression of SNARE protein, VAMP2, that is targeted in dnSNARE mice, was also analyzed regarding the OS of GBM patients. Patients with high expression levels of *VAMP2* (median OS = 404 days; 95 % CI = 353 - 454 days), presented a similar OS when compared with low expression levels of *VAMP2* (median OS = 430 days; 95 % CI = 387 - 472 days). No association was observed between the *VAMP2* expression and the patient OS (Cox p-value = 0.305). Moreover, similar analyses were performed regarding the expression of three additional SNARE complex genes (*VAMP3*, *SNAP23* and *SNAP25*) independently analyzed, but no significant association was found between the expression levels and OS of the patients (Supplementary Figure 2). Moreover, an analyses taking into consideration simultaneous the levels of the 5 SNARE proteins (VAMP2, VAMP3, SNAP 23, SNAP25, STX1A), revealed, that high SNARE expressers (median OS = 333 days; 95 % CI = 198 - 467 days) present a shorter OS when compared with low SNARE expression patients (median OS = 425 days; 95 % CI = 392 - 458 days; Cox p-value = 0.004; Figure 3.7). The results presented suggest clinical value regarding the expression of a SNARE proteins, indicating that the target of this complex can present relevance for GBM therapeutics.

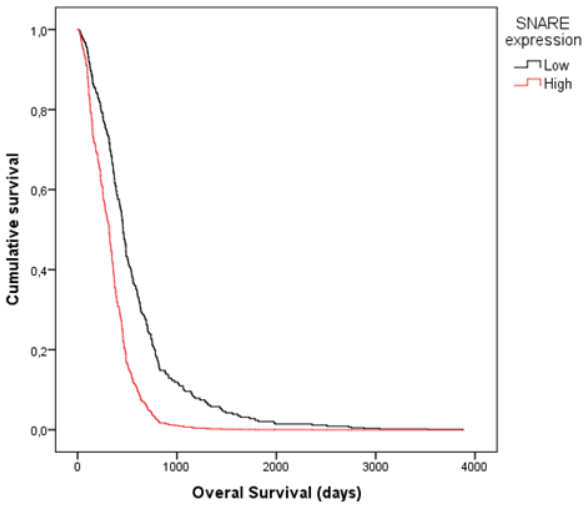
a



b

	Overall Survival	
	n	p-value (Cox)
Stx1A expression		
High	230	< 0.001
Low	159	
Gender		
Male	244	0.057
Female	145	
Chemotherapy		
Yes	334	0.876
No	55	
Radiotherapy		
Yes	354	< 0.01
No	35	
Age	387	< 0.01
KPS	387	< 0.01

c



d

	Overall Survival	
	n	p-value (Cox)
SNARE expression		
High	22	0.004
Low	367	
Gender		
Male	244	0.030
Female	145	
Chemotherapy		
Yes	334	0.747
No	55	
Radiotherapy		
Yes	354	< 0.01
No	35	
Age	387	< 0.01
KPS	387	< 0.01

Figure 3.7 - SNARE complex as a prognostic value in GBM patients - **(a)** Kaplan Meier survival curves of 554 GBM patients from TCGA regarding their STX1 expression levels **(b)** and respective multivariate survival analyses (cox model) **(c)** Kaplan Meier survival curves of 554 GBM patients from TCGA regarding their SNARE expression levels and respective multivariate survival analyses (cox model).**(d)** Multivariate analyses are adjusted for: gender, treatment with chemotherapy and radiotherapy, age and Karnofsky performance score (KPS)

Chapter 4 – Discussion

4 Discussion

Astrocytes are a major component of glioma microenvironment in brain parenchyma and their dysfunction is associated with several brain disorders. In this work, for the first time, the exocytosis mechanism of astrocytes was targeted, more precisely the vesicular release mediated by synaptobrevin II, and its impact was assessed on GBM pathophysiology. The *in vitro* results presented here suggest that astrocytes release factors that are able to increase glioma cells viability, an effect that was lost when astrocytes present a vesicular release impairment. Additionally, we observed that mice from both genotypes (WT and dnSNARE) developed tumors with similar morphological features and size. Interestingly, and despite of these observations, our *in vivo* results, suggest that mice with impaired astrocytic vesicular release have a better outcome in terms of survival after intracranial injection with glioma cells.

The paracrine regulation of glioma cells by astrocytes was firstly described in 2003, when the use of ACM lead to an increase in glioma proliferation and invasion (Le et al., 2003). Another studies revealed that the released factors by astrocytes, such as IL-6 or IGF-1, are directly associated with increased proliferation of cancer cells(Sierra et al., 1997). Moreover, some of the factors up-regulated in reactive astrocytes have been associated with the increase in glioma cells proliferation (reviewed in Placone et al., 2016). These results are supported by our findings, that showed an increase of glioma cells viability in the presence an increase in their viability in the presence of astrocytic CM. Specifically, we demonstrate here that this effect is only observed in the presence of CM derived from WT glial cultures when compared with control medium, which was not observed when used CM derived from dnSNARE glial cultures. We also demonstrated that it was not necessary a juxtacrine signaling by astrocytes for the increase in glioma cell viability, although it should not be discarded that this form of cellular communication might affect the results obtained. Supporting this idea, a study using lung metastatic cells identified a proliferative effect when lung cancer cells were co-cultured with astrocytes, but the effect was not present when they used ACM. Moreover, they demonstrated that astrocytes in contact with lung cancer cells change their secretory profile (Seike et al., 2011). A similar phenomenon is plausible to occur when astrocytes contact directly with glioma cells. The reactive state of astrocytes in the presence of brain injuries or cancer cells, result in the alteration of their secretory profile. Moreover, factors such as brain-derived neurotrophic factors (BDNF) that are released in dense-core vesicles by reactive astrocytes, and are diminished in CM from dnSNARE mice, could explain the results observed in cell viability of glioma cells (Xiong et al., 2013, Verkhratsky et al., 2016). Although viable cells counting and cell metabolic viability are associated with

cell proliferation, for a more suitable approach, a proliferative assay, as 5-Bromo-2-DeoxyUridine (BrdU) assay, should be performed to complement the *in vitro* data. Moreover, an additional complementary approach for this part of the work would be to target ectosomes, which are also released by astrocytes and contain MMPs and micro RNAs that are associated with an increased proliferation in glioma (Zhang et al., 2015). This approach constitutes another strategy to impair vesicular release by astrocytes, since our model affects mainly small and dense-core vesicles (Verkhratsky et al., 2016).

The roles of paracrine and juxtacrine regulation of glioma invasion by astrocytes has been recently explored (Placone et al., 2016). The assessment of GL261 cells migration when exposed to CM derived from WT or dnSNARE astrocytes results were inconclusive. Indeed, this glioma cell line susceptibility to confluent states in the absence of serum, can explain the lack of migration along the time observed in our migration assay. However, different studies present an increased invasion, on different glioma cell lines, when exposed to ACM using distinct migration/invasion assays, such as Boyden chamber assay (Le et al., 2003, Rath et al., 2013). The results of these studies suggest that the ACM lead to an increase in glioma invasion, a hallmark of GBM. Moreover, another study, revealed that the migration capacity was enhanced when glioblastoma stem-like cells were co-cultured with astrocytes, comparative to ACM, suggesting that cell-to-cell contact can be important for glioma cells migration (Rath et al., 2013). In lights of our results, it would be prudent to evaluate the glioma migration by a different invasion assay, and by using a dynamic co-culture system of glioma cells with astrocytes. Indeed, we believe that the use of different glioma cell lines would elucidate the variable sensibility of these heterogeneous tumors to ACM. Moreover, it would also be important to understand the changes in the secretory profile of astrocytes when in direct contact with cancer cells, as is the case of GBM patients, to better understand the role of paracrine and dynamic/longitudinal regulation of glioma cells by astrocytes, and vice-versa. The invasion profile gains even more importance considering that the tumor location is a crucial factor in GBM patient survival time (Mineo et al., 2007). A recent study revealed that astrocytes were able to promote glioma invasion via the gap junction protein connexin 43. They demonstrated by blocking gap junctions between astrocytes and glioma cells, mainly composed by Cx43, that the tumors presented a lower percentage of infiltrative edges (Sin et al., 2016). We hypothesize that the substances released by astrocytes in vesicles can directly impact the glioma cells invasion, presenting a similar role to the one attributed to gap junctions. Moreover, a different paracrine regulation of glioma invasion by astrocytes can happen by ECM modulation. In the case of brain injuries (*e.g.* tumors), astrocytes are able to secrete a variety of factors/proteins that remodel the tissue around the injury (Jones and Bouvier, 2014). Interestingly, in brain tumors, cancer cells are able to use these changes in ECM to invade the brain parenchyma. We

hypothesized that the results obtained regarding dnSNARE mice survival might be due to a more restricted ECM, since astrocytes do not release several factors in our model that can be implicated in ECM remodeling. The extracellular matrix glycoprotein TNC, secreted by reactive astrocytes, is highly expressed in the tumor microenvironment contributing for glioma invasion (Xia et al., 2016). In dnSNARE model, the TNC expression can be affected resulting on lower migration and consequently higher survival. However, these points regarding glioma migration should be further assessed, using the brain tissue of glioma bearing dnSNARE mice to evaluate the infiltrative edges of tumors and the expression of proteins associated with glioma invasion, as MMPs, being this hypothesis merely speculative at the moment.

Although our *in vitro* results suggest a role for astrocytic vesicular release, in terms of cell viability modulation, the translation for *in vivo* approaches and mostly for clinical therapeutics may be challenging. The *in vitro* results confirm that astrocytes have the capacity to secrete molecules that regulate glioma cells, although secretory system of astrocytes *in vivo* can present several differences. Depending on the type of brain cells surrounding the tumor area, specific types of interactions between them and the glioma cells may modulate the secretory profile of the astrocytes (Pekny and Pekna, 2014). Yet, the study of microenvironment on *in vivo* models has been mainly associated with the role of TAMs on glioma progression, specifically for molecules released by these cells (Markovic et al., 2009, Hambardzumyan et al., 2015).

Taking into account that astrocytes are the main glia cell in the brain, and their importance in several brain pathologies (Filous and Silver, 2016), we used a transgenic mouse model (dnSNARE) to explore the role of astrocytic vesicular release in GBM. Astrocytes react to the presence of neoplastic cells altering their morphology and secretory profile, as demonstrated by Nagashima and coworkers, where VEGF, MMP-2 and IL1-β were found to be strongly expressed in astrocytes around the tumor. Moreover, they also describe a high GFAP expression around the tumor from GBM patients, as observed in our histological analyses on tumor-bearing mice from both genotypes (Nagashima et al., 2002). Furthermore, our results indicate that the formation of tumor in the majority of the animals was independent of genotype, indicating that tumors had the conditions to develop even without the substances released by astrocytic vesicles. The features observed in our histological analyses, such as highly nuclear density and nuclear disorganization, are common features found in GBM patients, validating the use of our orthotopic model.

The *in vivo* outcomes presented here suggest that an impairment in astrocytic vesicular release seem to result in an increased survival for dnSNARE mice. However, this association was only observed after the assessment of GFP expression levels in dnSNARE mice. Different expression levels of GFP were

found between dnSNARE mice, correlating with dnSNARE transgene expression. The GFP levels were used to discriminate the dnSNARE genotype in two different groups. Taking into account that this transgenic model works as a competitive model between the VAMP2 cytosolic tail (transgene) and the endogenous VAMP2 (Pascual et al., 2005), it is plausible that astrocytes with a higher transgene expression present a higher impairment in astrocytic vesicular release. Considering that the levels of GFP expression were only accessed after the experiments, and after splitting it into two groups (high and low expressers), we obtained a low number of dnSNARE mice per group on *in vivo* experiments, that may explain the lack of statistical significance. Therefore, future studies should consider the repetition of this experiment.

Moreover, our results suggest that this survival outcome is not directly correlated with the tumor growth since no differences were found between tumor volume fourteen days after glioma cells injection. The lack of differences of tumor growth in our tumor-bearing mice can suggest possible compensatory mechanisms (eg increase in their secretory profile) from other non-neoplastic cells that directly interact with glioma cells. In fact, microglia is able to release several factors also released by astrocytes that have been already associated with tumor growth and invasion (Rock et al., 2004, Hambardzumyan et al., 2015). The transgenic mouse model used in our study specifically target GFAP positive cells, however our results point to an insufficient role for these cells at changing the growth profile of the tumor. Considering that microglia is able to secrete some factors released by astrocytes, we hypothesized that a compensatory mechanism can occur by reactive microglia in dnSNARE mice leading to similar tumor volumes in mice of both genotypes. Thus, it will be interesting to further assess the distribution of reactive microglia in both dnSNARE and control littermates. Supporting this hypothesis are the astrocyte-microglia interactions observed in the brain that appear to present an important role in the biology of these cells, by regulating their secretory profile (Rock et al., 2004). Furthermore, we need to take in consideration that dnSNARE expression only inhibits exocytosis mediated by VAMP2, meaning that astrocytes are still able to secrete factors through vesicles associated with different fusion proteins, as is the case of Rab GTPases (Verkhratsky et al., 2016). Beyond that, the modulatory molecules may be released by astrocytic transmembrane channels, which should be intact in the dnSNARE model. Finally, we know that some populations of astrocytes do not express dnSNARE (Sultan et al., 2015), and those astrocytes should behave within the brain parenchyma as WT astrocytes. Nevertheless, despite of possible compensatory mechanisms, the inhibition of astrocytic vesicular release was sufficient to induce a better outcome in terms of survival of the transgenic animals, suggesting that exocytosis-dependent regulation of ECM may be critical for GBM. By targeting complementary approaches, for instance, by acting through molecular

mechanisms as diffusion through membrane channels or translocation by plasmalemmal transport (Zorec et al., 2016), might result in a more effective treatment for GBM.

Another possible justification are the wide range of chemokine and cytokines secreted by astrocytes that act as immune mediators in cooperation with those produced by microglia, regulating the immune system at the CNS. The mechanism by which astrocytes secrete these immunological molecules is yet to be defined, but a release of these mediators by vesicles, similar to microglia secretion is plausible (Rock et al., 2004, Wang and Bordey, 2008, Claycomb et al., 2013). Although not described, it is possible that our dnSNARE model might have an impairment in the release of chemokines and cytokines, that are essential for an immune response. A critical step in the malignant progression of the tumor is the evasion and suppression of the host immune cells (Quail and Joyce, 2013). Several factors, that are upregulated in reactive astrocytes are associated with the immunosuppressive function that astrocytes can play in GBM. We hypothesize that the substances released in vesicles by astrocytes can have a direct role in the protection of tumor cells against the immune system. Considering that dnSNARE mice have an impairment in the release of those substances for the microenvironment, the astrocytic protective effect might be attenuated. Moreover, astrocytes release chemoattractant factors of TAMs that have been positive associated with tumor growth and invasion (Hambardzumyan et al., 2015). In fact, a recent study published this year demonstrated that astrocytes regulate PTEN expression in glioma cells by the release of microRNAs. The upregulation of PTEN result in the release of the TAMs chemoattractant CCL-2 leading to an increase population of microglia and TAMs around the tumor, consequently reducing the mice overall survival (Zhang et al., 2015). Astrocytes also secrete this chemokine by themselves (Carrillo-de Sauvage et al., 2012), and this feature can be compromised in our model, which might result in a lower infiltration by microglia and TAMs. Taking into account that the reduction of these cell types result in an increased survival of mice (Markovic et al., 2009), a similar phenomenon can explain the results here described. The assessment of TAMs and microglia population presence in our tumor-bearing mice brain tissue could support this hypothesis, and elucidate the *in vivo* results presented.

The target of astrocytes function in brain disorders is not a novel approach in clinics. Some molecules have been already tested in clinical trials, targeting the astrocytes-mediated glioma uptake that lead to neurodegeneration in stroke and ALS (Filous and Silver, 2016). A recent promising target to restrain glioma growth is related with the signal cues from reactive astrocytes in the tumor microenvironment. In brain tumors, astrocytes express endothelin (ET), a peptide with vasoconstrictive properties. The activation of both ET receptors was recently associated with a protective role against chemotherapy, by the upregulation of anti-apoptotic proteins in cancer cells. Moreover, blocking both ET

receptors with macitentan, a drug currently used for pulmonary arterial hypertension, abolished the astrocytic protection of glioma cells against chemotherapy. The treatment resulted in an increased mice survival and less tumor growth of tumor-bearing mice when treated with TMZ (Kim et al., 2015). This study opens the door for the direct target of astrocytes in the tumor microenvironment. Our results regarding STX1A (a SNARE complex protein) expression on TCGA, demonstrated that high expression levels of this gene are associated with a poor OS of GBM patients. This finding is extremely important, since using human GBM samples data we found that the expression levels of one single gene involved in the SNARE complex formation, was enough to affect the patients' overall survival. Takin into account that higher expression levels of this SNARE protein can suggest increased exocytosis, these results support the studies using the dnSNARE model, presented in this thesis. However, is necessary to considerer that mRNA levels analyzed in TCGA data base, cannot be directly associated with SNARE complex functionality. Moreover, the mRNA levels of STX1A are not exclusive of astrocytes, since there analyzed from hetero-cellular tumor samples collected from GBM patients. Although, STX1A was the only SNARE complex protein analyzed with prognostic value, we think that the target of a single SNARE protein, is enough to disrupt of the complex formation. In fact, this hypothesis is supported by the multivariate analyses considering the gene expression of 5 SNARE genes (*VAMP2; VAMP3; SNAP23; SNAP25; STX1A*), where the patients with a simultaneously high expression levels of the 5 genes, presented a shorter survival. We demonstrate here that the dysregulation of this complex could be a value target, for GBM treatment, considering their prognostic value. This results are supported by a Ulloa study, that reveal that the blockage of STX1A inhibits the glioblastoma tumor growth in a mice model (Ulloa et al., 2015), corroborating our data. The results presented during this thesis suggested that the target of SNARE complex specifically can be valid approach for the treatment of GBM. One possible approach could be the use of botulinum neurotoxins (BoNTs) that are currently used in the clinical to treat acute pain and Parkinson's disease (Jankovic et al., 2009). This drug is a selectively inhibitor of SNARE proteins, although not specific for astrocytes, that result in the impairment of SNARE complex. To overcome this problem, a viable approach should pass by the targeting of astrocytes sub-population around the tumor, stopping their supportive role. The target of this population can pass by the identification of specific combination/configurations of SNARE protein complex that are responsible for a diversity of exocytosis processes (Kasai et al., 2012).

In short, the role of astrocytes in glioblastoma can be essential for the disease progression. The study of astrocytes *in vivo* can be a challenge, due to the difficulty of targeting specifically these cells in models and patients. Considering that different cell types can secrete and express the same

proteins/genes, understanding the role of a single cell type is difficult. Here we demonstrate that a possible approach for the study of the cells role related to tumor microenvironment, can be performed by using transgenic mice models. The use of glioblastoma murine models, allows to mimic several interactions between the non-neoplastic cells and neoplastic cells observed in GBM patients. The treatment for GBM can pass by complementary approaches, of the current available treatment and the target of the protective role of the microenvironment.

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Chapter 5 – Conclusions and Future Perspectives

5 Conclusions and Future Perspectives

The results presented in this work suggest a role of the secreted molecules by astrocytic vesicles in GBM viability with a putative effect on tumor survival. However, the mechanisms of paracrine glioma regulation by astrocytes, should be further explored since the mechanisms involved in the increased of glioma viability observed here are still unknown at the moment. It will be interesting to characterize by proteomic analyses (*e.g. mass* spectrometry analysis), the CM derived from glial cultures obtained from both genotypes (WT vs dnSNARE) and assess the main glioma cell pathways that are activated/altered. Regarding the data presented during this thesis, a validation of our results using a distinct murine glioma cell line would be important. Moreover, additional functional analyses (*e.g.* as BrdU assay for proliferation, Annexin V / PI staining for apoptosis), would be a great addition to the *in vitro* work here presented. Regarding, the role of astrocytes in glioma migration, our results were inconclusive. Taking this into account, we propose the use of different invasion/migration assays (*e.g.* Boyden chamber assay) to confirm these results. Finally, all in vitro results were obtained using a static approach, using CM from glial cultures. A complementary approach with dynamic co-cultures of astrocytes with glioma cells could explore other possible roles of astrocytes in GBM progression, and understand the relevance of juxtacrine regulation of glioma cells by astrocytes.

Regarding the *in vivo* approach presented on this thesis, we revealed a possible role of astrocytes gliotrasnmission on GBM pathophysiology. Moreover, the tissues collected in our experiment from dnSNARE mice should be further explored regarding the distribution of reactive microglia and TAMs, based on their putative role in the disease progression. The brain tissue collected could also be used for identification of infiltrative edges present in the tumor, exploring some of the hypothesis debated during the results discussion. Considering that the target of this astrocytic mechanism in patients, will be probably result in secondary effects, as a cognitive decline. Thus, we propose to explore this astrocytic inhibition using a complementary model. The use of virus with BoNTs specifically targeting astrocytes could support and increase the relevance of the results present during this thesis, since it would locally target the SNARE complex of astrocytes in a specific brain region. Finally, a micro-array analysis of tumor tissue derived from WT and dnSNARE mice will be relevant regarding the understanding of GBM mechanisms and cell pathways regulation, mediated by astrocyte exocytosis associated to VAMP2.

It is my belief that only a multi targeting of different regulatory mechanism of GBM, including their microenvironment, will result in better treatments with a significantly increase on the average life expectancy of GBM patients.

Chapter 6 – References

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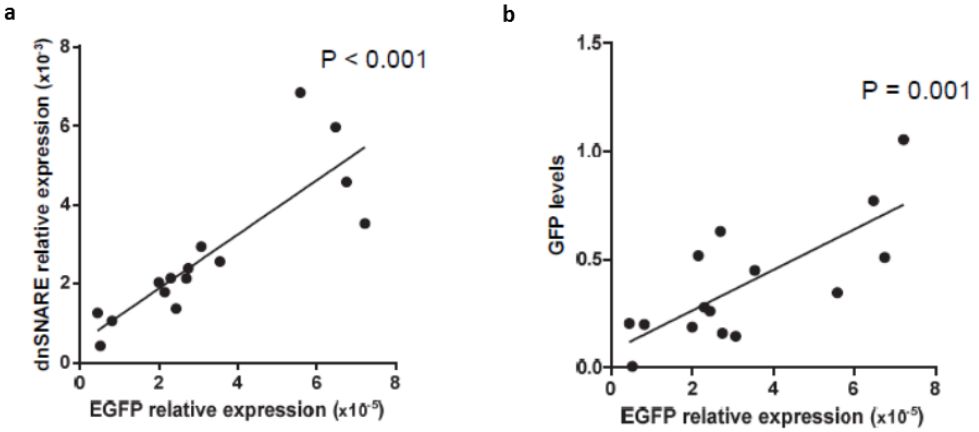
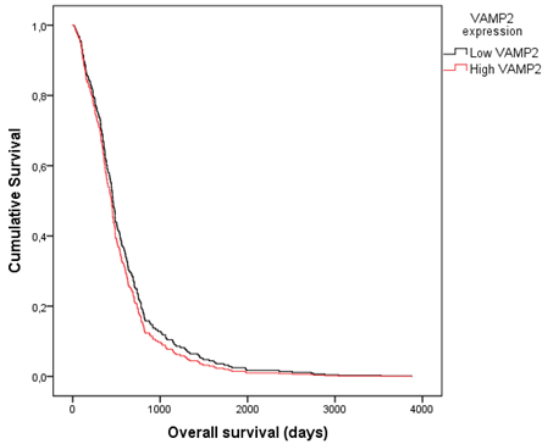
Chapter 7 – Supplementary Information

7 Supplementary Information



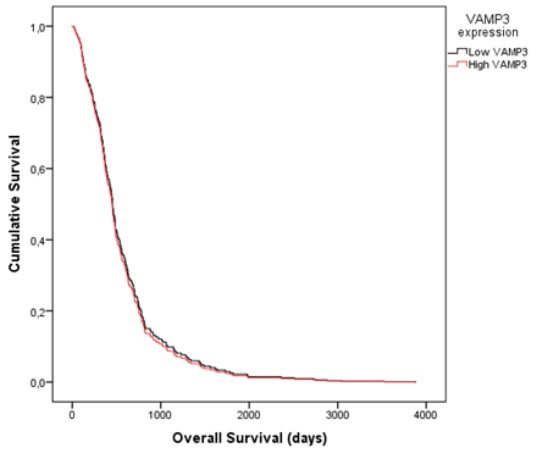
Supplementary Figure 1 – GFP reporter is a good readout of dnSNARE transgene expression - Direct correlation between expression levels of EGFP mRNA and dnSNARE mRNA **(a)**, or GFP protein levels **(b)** measure in the same set of dnSNARE mice (data not published).

a



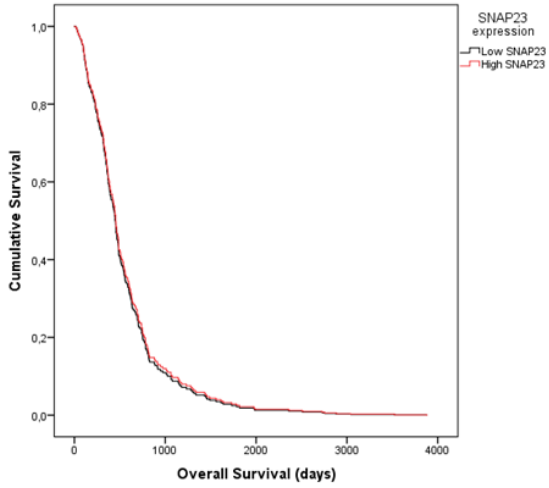
	Overall Survival	
	n	p-value (Cox)
Vamp2 expression		
High	193	0.305
Low	196	
Gender		
Male	244	0.046
Female	145	
Chemotherapy		
Yes	334	0.714
No	55	
Radiotherapy		
Yes	354	< 0.01
No	35	
Age	387	< 0.01
KPS	387	< 0.01

b



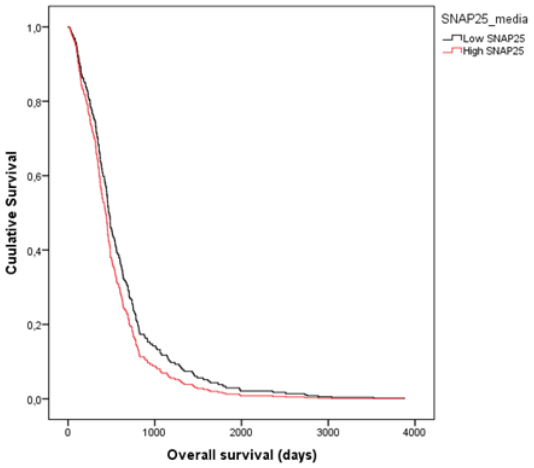
	Overall Survival	
	n	p-value (Cox)
Vamp3 expression		
High	202	0.686
Low	187	
Gender		
Male	244	0.040
Female	145	
Chemotherapy		
Yes	334	0.594
No	55	
Radiotherapy		
Yes	354	< 0.01
No	35	
Age	387	< 0.01
KPS	387	< 0.01

c



	Overall Survival	
	n	p-value (Cox)
SNAP23		
High	199	0.731
Low	190	
Gender		
Male	244	0.044
Female	145	
Chemotherapy		
Yes	334	0.648
No	55	
Radiotherapy		
Yes	354	< 0.01
No	35	
Age	387	< 0.01
KPS	387	< 0.01

d



	Overall Survival	
	n	p-value (Cox)
SNAP25 expression		
High	207	0.070
Low	182	
Gender		
Male	244	0.069
Female	145	
Chemotherapy		
Yes	334	0.749
No	55	
Radiotherapy		
Yes	354	< 0.01
No	35	
Age	387	< 0.01
KPS	387	< 0.01

Supplementary Figure 2 - Relation between SNARE proteins and OS of GBM patients – Kaplan Meier survival curves of 554 GBM patients from TCGA regarding **(a)** VAMP2; **(b)** VAMP3; **(c)** SNAP23 and **(d)** SNAP25 mRNA expression levels, and respective multivariate analyses (adjusted for: gender, treatment with chemotherapy and radiotherapy, age and KPS).